

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
 US Department of Commerce
 United States Patent and Trademark
 Office, PCT
 2011 South Clark Place Room
 CP2/5C24
 Arlington, VA 22202
 ETATS-UNIS D'AMERIQUE
 in its capacity as elected Office

Date of mailing (day/month/year) 11 July 2001 (11.07.01)	
International application No. PCT/US00/26504	Applicant's or agent's file reference 44508-5003WO
International filing date (day/month/year) 27 September 2000 (27.09.00)	Priority date (day/month/year) 27 September 1999 (27.09.99)
Applicant DALY, Michael, J. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

25 April 2001 (25.04.01)

☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer Nestor Santesso Telephone No.: (41-22) 338.83.38
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/26504

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 1/20; A01N 63/00

US CL : 435/252.3; 424/93.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/252.3; 424/93.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, MEDLINE, CAPLUS, EMBASE, BIOSIS

search terms: Michael Daly, Lawrence Wackett, deinococcus, bph gene, radiation, resistant, bacteria

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CAI, L. et al. Metallothionein in radiation exposure: its induction and protective role. February 1999. Vol. 132, No. 2-3. pages 85-98. See entire article.	1-4, 6, 13-16, 18, 25, 26
X	LANGE, C.C. et al. Engineering a recombinant Deinococcus radiodurans for organopollutant degradation in radioactive mixed waste environments. Nature Biotechnology. October 1998. Vol. 16, No. 10. pages 929-933. See entire article.	1-4, 7-16, 19-41
Y		5,6,17,18
Y	SMITH, M. et al. Gene expression in Deinococcus radiodurans. Gene. 1991. Vol. 98. pages 45-52. See entire article.	1-31, 35-41

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* & * document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 04 DECEMBER 2000	Date of mailing of the international search report 12 MAR 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PETER TUNG Telephone No. (703) 308-0196 TERRY J. DEY PARALEGAL SPECIALIST TECHNOLOGY CENTER 1600

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/26504

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DOWLING, D. et al. A DNA module encoding bph genes for the degradation of polychlorinated biphenyls. FEMS Microbiology Letters. 1991. Vol. 113. pages 149-154. See entire article.	7,11,12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/26504

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 32-34
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

RECEIVED

PATENT COOPERATION TREATY

RGA
JST
RJS
PF

JUL 19 2001

MORGAN, LEWIS & BOCKIUS LL PCT

From the INTERNATIONAL BUREAU

To:

MORGAN, LEWIS & BOCKIUS LLP
1800 M Street, NW
Washington, DC 20036
ETATS-UNIS D'AMERIQUEINFORMATION CONCERNING ELECTED
OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

Date of mailing (day/month/year)

11 July 2001 (11.07.01)

Applicant's or agent's file reference

44508-5003WO

IMPORTANT INFORMATION

International application No.

PCT/US00/26504

International filing date (day/month/year)

27 September 2000 (27.09.00)

Priority date (day/month/year)

27 September 1999 (27.09.99)

Applicant

THE HENRY M. JACKSON FOUNDATION et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

EP : AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE

National : AU, BG, CA, CN, CZ, DE, IL, JP, KP, KR, MN, NO, NZ, PL, RO, RU, SE, SK, US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

AP : GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW

EA : AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

OA : BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

National : AE, AG, AL, AM, AT, AZ, BA, BB, BR, BY, BZ, CH, CR, CU, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IN, IS, KE, KG, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MW, MX, MZ, PT, SD, SG, SI, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW

3. The applicant is reminded that he must enter the "national phase" before the expiration of 30 months from the priority date before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing, if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

The entry into the European regional phase is postponed until 31 months from the priority date for all States designated for the purposes of obtaining a European patent.

DOCKETED

By

Date

7/19/01

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer:

Nestor Santesso

Facsimile No. (41-22) 740.14.35

Telephone No. (41-22) 338.83.38

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PCT/US00/26504

JAN 22 2001

PATENT COOPERATION TREATY

MORGAN, LEWIS & BOCKIUS LLP

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

MORGAN, LEWIS & BOCKIUS LLP
1800 M Street, NW
Washington, DC 20036
ETATS-UNIS D'AMERIQUEDate of mailing (day/month/year)
09 January 2001 (09.01.01)Applicant's or agent's file reference
44508-5003WO

IMPORTANT NOTIFICATION

International application No.
PCT/US00/26504International filing date (day/month/year)
27 September 2000 (27.09.00)International publication date (day/month/year)
Not yet publishedPriority date (day/month/year)
27 September 1999 (27.09.99)

Applicant

THE HENRY M. JACKSON FOUNDATION et al

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.
3. An asterisk(*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
4. The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
27 Sept 1999 (27.09.99)	60/155,767	US	27 Dec 2000 (27.12.00)

DOCKETED

By GF Date 1/22/01The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer

Magda BOUACHA

Facsimile No. (41-22) 740.14.35

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

WO 01/23526
PCT/US00/26504

RGA
MST

PCT

From the INTERNATIONAL BUREAU

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
MORGAN, LEWIS & BOCKIUS LLP
1800 M Street, NW
Washington, DC 20036
ETATS-UNIS D'AMERIQUE

RECEIVED

APR 16 2001

MORGAN, LEWIS & BOCKIUS

Date of mailing (day/month/year) 05 April 2001 (05.04.01)		IMPORTANT NOTICE	
Applicant's or agent's file reference 44508-5003WO			
International application No. PCT/US00/26504	International filing date (day/month/year) 27 September 2000 (27.09.00)	Priority date (day/month/year) 27 September 1999 (27.09.99)	
Applicant THE HENRY M. JACKSON FOUNDATION et al			

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
AU,KP,KR,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

AE,AG,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CR,CU,CZ,DE,DK,DM,DZ,EA,EE,EP,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,
The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
05 April 2001 (05.04.01) under No. WO 01/23526

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

DOCKETED

By GI Date 7/16/01

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

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PATENT COOPERATION TREATY

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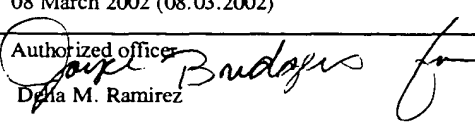
INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 16 APR 2002

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Applicant's or agent's file reference 44508-5003WO		FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/26504	International filing date (day/month/year) 27 September 2000 (27.09.2000)	Priority date (day/month/year) 27 September 1999 (27.09.1999)	
International Patent Classification (IPC) or national classification and IPC IPC(7): C12N 1/20; A01N 63/00 and US Cl.: 435/252.3; 424/93.2			
Applicant THE HENRY M. JACKSON FOUNDATION			
<p>1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of <u>5</u> sheets, including this cover sheet.</p> <p><input type="checkbox"/> This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).</p> <p>These annexes consist of a total of <u>0</u> sheets.</p>			
<p>3. This report contains indications relating to the following items:</p> <p>I <input checked="" type="checkbox"/> Basis of the report</p> <p>II <input type="checkbox"/> Priority</p> <p>III <input checked="" type="checkbox"/> Non-establishment of report with regard to novelty, inventive step and industrial applicability</p> <p>IV <input type="checkbox"/> Lack of unity of invention</p> <p>V <input checked="" type="checkbox"/> Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p>VI <input type="checkbox"/> Certain documents cited</p> <p>VII <input type="checkbox"/> Certain defects in the international application</p> <p>VIII <input checked="" type="checkbox"/> Certain observations on the international application</p>			
Date of submission of the demand 25 April 2001 (25.04.2001)		Date of completion of this report 08 March 2002 (08.03.2002)	
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230		Authorized officer  Delia M. Ramirez Telephone No. (703) 308-0196	

Form PCT/IPEA/409 (cover sheet)(July 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/26504

I. Basis of the report

1. With regard to the elements of the international application:*

☒ the international application as originally filed.☒ the description:

pages 1-49 as originally filed

pages NONE, filed with the demand

pages NONE, filed with the letter of _____.

☒ the claims:

pages 50-55, as originally filed

pages NONE, as amended (together with any statement) under Article 19

pages NONE, filed with the demand

pages NONE, filed with the letter of _____.

☒ the drawings:

pages 1-15, as originally filed

pages NONE, filed with the demand

pages NONE, filed with the letter of _____.

☐ the sequence listing part of the description:

pages NONE, as originally filed

pages NONE, filed with the demand

pages NONE, filed with the letter of _____.

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).☐ the language of publication of the international application (under Rule 48.3(b)).☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 a.d/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.☐ filed together with the international application in computer readable form.☐ furnished subsequently to this Authority in written form.☐ furnished subsequently to this Authority in computer readable form.☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.4. ☒ The amendments have resulted in the cancellation of:☒ the description, pages NONE☒ the claims, Nos. NONE☒ the drawings, sheets/fig NONE5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/26504

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The question whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application,

☒ claims Nos. 32-34

because:

☐ the said international application, or the said claim Nos. _____ relate to the following subject matter which does not require international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. _____ are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 32-34

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. STATEMENT**

Novelty (N)	Claims <u>6,10,18,22,29,30,35-40</u>	YES
	Claims <u>1-5,7-9,11-17,19-21,23-28,31,41</u>	NO
Inventive Step (IS)	Claims <u>NONE</u>	YES
	Claims <u>1-31, 35-41</u>	NO
Industrial Applicability (IA)	Claims <u>1-31, 35-41</u>	YES
	Claims <u>NONE</u>	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-5, 7-9, 11-17, 19-21, 23-28, 31 and 41 lack novelty under PCT Article 33(2) as being anticipated by Lange et al. Lange et al. teaches the radiation resistant bacterium *D. radiodurans* comprising DNA encoding toluene dehydrogenase. The reference teaches that *D. radiodurans* detoxifies toluene, chlorobenzene and 3,4-dichloro-1-butene when grown in the presence of these compounds.

Claims 6, 18, 29, 30, 35-40 lack an inventive step under PCT Article 33(3) as being obvious over Lange et al. in view of Philippidis et al. The teachings of Lange et al. have been discussed supra. Lange et al. also teaches that microorganisms can be used to clean up waste sites containing high levels of organopollutants, radionuclides, and heavy metals. Lange et al. does not teach a radiation resistant bacterium which detoxifies heavy metals. Philippidis et al. teach that the *E. coli merA* gene product encodes a mercuric reductase which provides for resistance to mercury. Philippidis et al. does not teach a radiation resistant bacterium. It would have been obvious for one of ordinary skill in the art to transform *D. radiodurans* containing DNA encoding putida toluene oxidase, as taught by Lange et al., with DNA encoding the *merA* mercuric reductase, as taught by Philippidis et al., for the benefit of a bacterium resistant to organopollutants, radionuclides, and heavy metals. One of ordinary skill in the art would have a reasonable expectation of success at doing this as bacteria expressing several heterologous DNAs is well known in the art. Therefore, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made.

Claims 10 and 22 lack an inventive step under PCT Article 33(3) as being obvious over Lange et al. in view of Dowling et al. The teachings of Lange et al. have been discussed supra. Lange et al. also teaches that microorganisms can be used to clean up waste sites containing high levels of organopollutants which include polychlorinated biphenyls. Lange et al. does not teach a radiation resistant bacterium which detoxifies polychlorinated biphenyls. Dowling et al. teaches that polychlorinated biphenyls may be removed from the environment by microbial detoxification. The reference further teaches DNA encoding enzymes which can catabolize polychlorinated biphenyls. Dowling et al. does not teach a radiation resistant bacterium. It would have been obvious at the time the invention was made for one of ordinary skill in the art to transform radiation resistant bacterium *D. radiodurans*, as taught by Lange et al., with DNA encoding the enzymes which catabolize polychlorinated biphenyls. One of ordinary skill in the art would have been motivated to combine the two references as Lange et al. teaches that polychlorinated biphenyls are found in waste sites and Dowling et al. teaches DNA encoding enzymes which catabolize polychlorinated biphenyls. One of ordinary skill in the art would have a reasonable expectation of success at doing this as bacteria expressing heterologous DNAs is well known in the art. Therefore, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art at the time of the invention.

Claims 1-31 and 35-41 meet the criteria for industrial applicability as defined by PCT Article 33(4) because the claimed invention is drawn to genetically engineered microorganisms that can degrade polychlorinated biphenyls. Such microorganisms can be used to detoxify sites contaminated with polychlorinated biphenyls, which are very harmful organopollutants.

----- NEW CITATIONS -----

PHILIPPIDIS et al. Effect of gene amplification on mercuric ion reduction activity of *Escherichia coli*. Appl. Environ. Microbiol. December 1991, Vol 57, pages 3558-3564.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/26504

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the questions whether the claims are fully supported by the description, are made:

Claims 10 and 22 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because claims 10 and 22 are indefinite for the following reason(s): the claims contain the trade name Arochlor.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
5 April 2001 (05.04.2001)

PCT

(10) International Publication Number
WO 01/23526 A1

(51) International Patent Classification⁷: C12N 1/20, A01N 63/00

(21) International Application Number: PCT/US00/26504

(22) International Filing Date:
27 September 2000 (27.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/155,767 27 September 1999 (27.09.1999) US

(71) Applicant (for all designated States except US): THE HENRY M. JACKSON FOUNDATION [US/US]; 1401 Rockville Pike, Suite 600, Rockville, MD 20852 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): DALY, Michael, J. [US/US]; 1401 Rockville Pike, Suite 600, Rockville, MD 20852 (US). WACKETT, Lawrence, P. [US/US]; 1401 Rockville Pike, Suite 600, Rockville, MD 20852 (US).

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(54) Title: ENGINEERED RADIATION RESISTANT BIOREMEDIATING BACTERIA

(57) Abstract: The invention relates to radiation resistant bacteria engineered to detoxify at least one toxin and preferably several toxins. Radiation resistant bacteria of the invention include *Deinococcus* strains engineered to detoxify toxins such as radionuclides, heavy metals and organic compounds. The invention also includes bioremediation compositions comprising at least one radiation resistant bacterial strain capable of detoxifying toxins and methods relating to the preparation and use of such compositions. Such compositions and methods may be utilized to detoxify industrial waste sites contaminated with radioactivity.

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ENGINEERED RADIATION RESISTANT BIOREMEDIATING BACTERIA

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FIELD OF THE INVENTION

The invention relates generally to the production of radiation resistant
10 microorganisms which are useful bioremediation agents. Preferred microorganisms include *Deinococcus* species, including *D. radiodurans* and *D. geothermalis* strains that have been engineered to metabolize, degrade or detoxify inorganic and organic contaminants such as radionuclides, heavy metals and organic solvents. This application claims priority to U.S. Provisional Application 60/155,767, filed September 27, 1999
15 which is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Many of the solid and liquid wastes generated as a result of global nuclear weapons production between 1945 and 1986 were discharged to the ground and are now contaminating the subsurface at many sites. These wastes contain inorganic and organic
20 contaminants that include radionuclides, heavy metals, acids/bases, and solvents (Riley *et al.*, 1992). In the United States alone, it is estimated that these leaking buried wastes ($3 \times 10^6 \text{ m}^3$) have contaminated $7.5 \times 10^7 \text{ m}^3$ of surface and subsurface soils and about $2 \times 10^{12} \text{ dm}^3$ of groundwater (Office of Energy Research, DOE, 1992). With the end of the Cold War in the early 1990's, the United States Department of Energy (DOE) shifted its
25 emphasis from nuclear weapons production to cleanup of its radioactive waste. This remediation effort is now the largest program of its kind ever undertaken by the United States (Macilwain, 1996).

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In 1992, the DOE surveyed a representative 91 out of 3,000 contaminated sites at eighteen U.S. research facilities (Riley *et al.*, 1992). Site-characterization activities at those 91 sites have recorded large inventories of chemical and radioactive contaminants in the soils, sediments and ground waters surrounding these disposal sites (Riley *et al.*,
5 1992). The most common metallic contaminants from DOE wastes that have been found in ground waters include the radionuclides $^{235}\text{Uranium}$ (γ , α)^E, $^{90}\text{Strontium}$ (β^-)^E, $^{238}\text{Plutonium}$ (α)^E, $^{137}\text{Cesium}$ (γ , β^-)^E, and $^{99}\text{Technetium}$ (β^-)^E; and the metals, Chromium, Lead and Mercury (Riley *et al.*, 1992, McCullough *et al.*, 1999). A more limited soil/sediment characterization has shown these same constituents throughout soil profiles
10 and sediments (Riley *et al.*, 1992). One third of the ninety-one sites are radioactive with some reported radiation levels as high as 10 mCi/L, within or close to the contaminating source. These high radiation levels are extremely damaging to living organisms over extended periods, often resulting in cell death.

Of the 3,000 waste sites disclosed by DOE, the total cleanup cost, by
15 physicochemical methods, was estimated in 1988 to be about \$90 billion (U.S. Government Accounting Office, GAO, 1988) and more recently between \$189 and \$265 billion, over a seventy year period (1996 Baseline Environmental Management Report (visited September 27, 1999) <<http://www.em.doe.gov/bemr96/>>). DOE budget projections for cleanup activities for just the next ten years exceed \$60 billion
20 (McCullough *et al.*, 1999). These sites, therefore, represent defined targets for less expensive *in situ* bioremediation technologies utilizing specialized microorganisms that can remediate both metallic and organic contaminants. The utility of microbiological methods for the treatment of highly radioactive waste environments will largely be determined by the ability of microorganisms catalyzing the desired function(s), to survive
25 and function under radiation stress.

Numerous microorganisms (including *Shewanella*, *Geobacter* and *Pseudomonas* spp.) have been described, and studied in detail, for their ability to transform, detoxify, or immobilize, a variety of organic and metallic pollutants (Gorby *et al.*, 1992; Higham *et al.*, 1984; Ji *et al.*, 1992; Lovely, 1995; Nies *et al.*, 1995; Tsapin *et al.*, 1996; Turner *et al.*,
30 1995; Voordouw *et al.*, 1996). Detoxification of the toxic compounds and metals at these

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sites is an important goal in remediating or stabilizing contaminated sites as well as preventing further dissemination. Generally, microorganisms are sensitive to the damaging effects of ionizing radiation, and most of the bacteria currently being studied as candidates for bioremediation are no exception. For example, *Pseudomonas* spp. is very sensitive to radiation (more sensitive than *E. coli* [Thornley, 1963]) and is not suited to remediate radioactive wastes. Therefore, radiation resistant microorganisms that can remediate toxic metals need to be identified in nature or engineered in the laboratory to address this problem.

SUMMARY OF THE INVENTION

10 The present invention is based in part on the discovery that the most radiation resistant organism yet discovered, *Deinococcus*, can be engineered to express heterologous enzymes capable of detoxifying or metabolizing organic compounds, heavy metals and radionuclides.

The invention includes radiation resistant bacteria engineered to detoxify at least one toxin, preferably radiation resistant strains which survive acute exposure to ionizing radiation of up to about 15,000 Gy or can grow in the presence of continuous radiation of about 60 Gy/hour, most preferably, radiation resistant strains of *Deinococcus* engineered to detoxify at least one toxin, such as radionuclides, heavy metals and organic compounds.

The invention also includes radiation resistant bacterial strains engineered to detoxify at least two toxins. Radiation resistant bacteria of the invention include *Deinococcus* strains engineered to express a heterologous protein or enzyme selected from the group consisting of toluene dioxygenase, the proteins encoded by the *mer* operon, the proteins encoded by the *Pseudomonas tol* region, the proteins encoded by the *xylL-xyIE* operon, a monooxygenase, the proteins encoded by *bphA1A2A3A4*, the proteins encoded by *czcA*, B and C genes, the proteins encoded by a *cytC3*, the protein encoded by the *smtA* *abdB* genes and the *arsA* and B genes

The invention also includes bioremediation compositions comprising at least one radiation resistant bacterial strain of the invention. Such bioremediation compositions may contain, in addition to the bacterial strains of the invention and other compounds or

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diluents, agents selected from the group consisting of film forming agents and nutrient agents. Bioremediation compositions of the invention may also be formulated for controlled release.

A further embodiment of the invention includes methods of bioremediation, comprising the step of exposing a sample to a bioremediating composition of the invention. The compositions of the invention may also be released into an industrial or other waste site.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Regional chromosomal maps and functions of *D. radiodurans* strains MD417 (*tod*⁻) and MD560 (*tod*⁺). Construction of these strains is described in the Experimental Protocol. MD560 constitutively expresses TDO (encoded by *todC1C2BA*). MD417 is a control strain (lacking *tod* genes). The strains are the products of transformation of wild-type strain R1 with the circular plasmids pMD532 and pMD417, respectively. The two arrows drawn between chromosomal regions MD417 and MD560 show the location of the *tod* genes; the black arrow below the *tod* genes shows the direction of functional transcription. The checkered segment **BC** indicates the chromosomal integration sequence. **A** and **D** are chromosomal sequences flanking the integration site **BC**. Km (resistance to kanamycin) is encoded by the *aphA* gene [diagonally hatched segment]. Transcription of the *aphA* genes is driven by a *Deinococcal* constitutive promoting sequence (open arrow) located in the black segments. Tc (resistance to tetracycline) is encoded by the *tet* gene [white region]. Transcription of the *tet* and *todC1C2BA* genes is driven by another *Deinococcal* constitutive promoting sequence (open arrow) present in the light grey segments. Restriction sites: X, *Xba*I; B, *Bam*HI; E, *Eco*RI.

Figure 2. Effect of γ -irradiation on the growth of *E. coli* (left) and *D. radiodurans* R1 (right). *E. coli* (wildtype) and *D. radiodurans* R1 (wildtype) were both grown to the

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plateau phase of their respective growth cycles and diluted 150-fold in fresh growth media. The diluted cultures were divided into two parts and incubated with aeration in the presence and absence of γ -radiation (^{137}Cs ; 60 Gy/hr) for a total of thirty hours. The survival rates were determined by plating appropriate dilutions of irradiated cells and counting the number of colony forming units (cfu) following incubation.

Figure 3. Effect of γ -irradiation on the synthesis and function of TDO expressed in *D. radiodurans*. Strain MD560 (*tod*⁺) in the presence of (solid triangle) and absence of (open circle) irradiation; and MD417 (*tod*⁺) in the presence of (solid circle) and absence of (open triangle) irradiation. Initially, strains MD560 and MD417 were grown in the presence and absence of γ -irradiation (60 Gy/hr) for sixteen hours to the plateau phase, in the absence of chlorobenzene. Cultures were then diluted with fresh medium and exponentially growing cells were harvested following continued growth in the presence and absence of γ -radiation (60 Gy/hr). Cells were then concentrated and incubated with 125 μM chlorobenzene in the presence and absence of irradiation (60 Gy/hr) for the indicated time periods.

Figure 4. Incubation of ^{14}C -labeled toluene with *D. radiodurans* strains MD560 (*tod*⁺; closed triangle) and MD417 (*tod*⁺; open square), *E. coli* (pDTG351; *tod*⁺; solid square), and negative control of TGY medium alone (open circle). Detection of ^{14}C non-volatile product is as described in Experimental Protocol.

Figure 5. Detection of non-volatile ^{14}C -labeled material in media containing cells after an 18-hour incubation with ^{14}C -trichloroethylene. *D. radiodurans* strain MD560 (*tod*⁺; black), MD417 (*tod*⁺, open), and TGY medium control (grey).

Figure 6. Effect of toluene and TCE on the growth of *D. radiodurans* strains R1, MD417 (vector control, *tod*⁺) and MD560 (*tod*⁺). Strains were first grown overnight in liquid growth medium (to 1.1 OD₆₀₀) followed by dilution into fresh growth medium (to 0.02 OD₆₀₀) containing varying amounts of toluene (left) and TCE (right). After eighteen hours

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of incubation at 32°C, the cell densities of the cultures were determined and plotted as a function of solvent concentration.

Figure 7. Plasmid and chromosomal maps. Top, 4.2 kb *mer* operon of pBD7 (Barrineau *et al.*, 1984) encoding six proteins: MerR, activation/repression of the *mer* operon; MerT, mercuric ion transport protein; MerP, periplasmic mercuric ion binding protein; MerC, transmembrane protein; MerA, mercuric reductase; and MerD, putative secondary regulatory protein. OP, operator/ promoter sequence. **A)** The *Stu*I site at the end of the *mer* operon was converted to a *Bgl*II (Bg) site, yielding pMD725, followed by the conversion of the *Nco*I (N) site at the start of the operon to an *Mfe*I (M) site, yielding pMD726. The *Mfe*I-*Bgl*II (4.2 kb) fragment of pMD726, was cloned into the *Eco*RI (E)-*Bam*HI (B) site of the *D. radiodurans* plasmid pMD66 (Daly *et al.*, 1994a), yielding pMD727. pMD727 was transformed into *D. radiodurans* strain R1 (wild-type) by selection with kanamycin (Km), giving strain MD735. The specifics of DNA cloning, Southern blotting and transformations were as described in the Experimental section.

B) dORI, *Deinococcal* origin of replication (Daly *et al.*, 1994b; 1995; 1996; 1997). eORI, *E. coli* origin of replication (Daly *et al.*, 1994a). P1 and P2 are two different constitutive *Deinococcus* promoters (Lange *et al.*, 1998). Km^R, kanamycin resistance gene *aphA*. **B)** The *Mfe*I-*Bgl*II (4.2 kb) fragment of pMD726 was cloned into the *Eco*RI-*Bam*HI site of the *D. radiodurans* tandem duplication vector pMD417 (Daly *et al.*, 1996), yielding pMD728. pMD728 was transformed into *D. radiodurans* strain R1 with Km selection, giving strain MD736. Two rounds of recombinative duplication are illustrated, yielding two vector copies on a chromosome. bc, duplicated chromosomal target sequence. X, *Xba*I. Remaining abbreviations and symbols are as in A. **C)** The unique *Dra*I site of the *D. radiodurans* amplification vector pS11 (Smith *et al.*, 1988) was converted to an *Nco*I site, yielding pMD729. The *Nco*I-*Bgl*II fragment of pMD725 was cloned into the *Nco*I-*Bgl*II site of pMD729, yielding pMD731. pMD731 was transformed into *D. radiodurans* strain R1 with Km selection, giving strain MD737. Multiple rounds of recombinative duplication are illustrated, yielding many insertions per chromosome. abcd, duplicated chromosomal target sequence. Remaining abbreviations and symbols are as in A.

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Figure 8. Construction and structure of a chromosomal direct insertion of the *mer* operon.

- A) Strain MD399 (Daly *et al.*, 1995) is a previously constructed *D. radiodurans* strain containing a direct insertion of a plasmid having regions of identity with the duplication insertion in strain MD736. ABC and DEF are contiguous chromosomal sequences in wildtype *D. radiodurans* strain R1, lacking homology. BC is the duplicated chromosomal flanking region in MD736. Open-headed arrows are constitutive deinococcal promoters (Lange *et al.*, 1998). Black crosses between the MD399 and MD736 chromosomes link regions of homology and show where crossovers occurred. Cm^R, chloramphenicol resistance gene, *cat*. Km^R, kanamycin resistance gene, *aphA*. Tc^S, mutated tetracycline gene (Daly *et al.*, 1994b), *tet*. E, *EcoRI*; P, *PvuII*; X, *XbaI*; Xh, *XhoI*; B, *BamHI*; P/Sy, *PvuII/StyI* fusion. *mer*, 4.2 kb *mer* operon. B) The standard transformation protocol (Daly *et al.*, 1994a) was used to introduce MD736 DNA into MD399. However, following the addition of transforming MD736 DNA and overnight incubation with MD399, 0.1 ml of the transformed cell suspension (~1 x 10⁷ cells) were transferred to 0.9 ml fresh TGY liquid medium containing 15 µg/ml Merbromin. After an eighteen hour incubation with shaking at 32°C, aliquots of 100 µl of transformed cells were spread on petri plates of non-selective TGY solid medium (30 cm³/plate). Once dry, 8 µl of 0.1 M Merbromin were pipetted onto the center of the plate. Mercury-resistant colonies grew, and were isolated from, within a zone of wildtype growth inhibition. MD399 (left, control); MD399 + MD736 DNA (right). C) Right, MD767 was selected and subjected to a detailed mapping of the *mer* operon integration site using restriction enzymes, Southern blotting, and probing with various radiolabeled DNA fragments, including a probe made from the *EcoRI-BglIII* fragment of the *mer* operon (black wavy line). Left, The chromosomal structure of the direct chromosomal insertion containing the *mer* operon in MD767.
- Abbreviations and symbols are as described in A.

Figure 9. Determination of *mer* operon copy number and associated mercury resistance phenotype. A) Genomic DNA from each of the exponentially growing strains R1, MD735, MD767, MD736, MD737 was prepared as described in the Experimental section and previously described (Daly *et al.*, 1994a). For BL308 (*E. coli* strain K12/pDB7) genomic

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DNA was prepared from stationary-phase cells. ~600 ng of each DNA sample was cut with *EcoRI* and electrophoresed at 60 volts for sixteen hours in a 0.6 % agarose gel. λ /H, lambda phage DNA cut with *HindIII*. **B**) The gel was blotted and hybridized to a radiolabeled 1.5 kb *EcoRI*-*BglIII* fragment of pMD726, containing part of *merA* and all of *merD*. The order of lanes is as in A. The number of disintegration counts in each of the hybridizing bands was determined using the Instant Imager (Packard Instrument Company) and adjusted for DNA content present in each of the corresponding gel lanes (A), similarly scanned. **C**) Genomic DNA was prepared from strains MD767 (direct insertion) and MD736 (tandem duplication) growing in increasing concentrations of Merbromin (0-35 μ M in 5 μ M steps, lanes 1-8, respectively). DNA was analyzed as described in A and B. The 23 kb, 9.4 kb and 6.5 kb λ /H size markers are shown. **D**) Growth curves for each of the strains described in A and B were determined by inoculating ~5 x 10⁶ cells of each into growth medium containing 50 μ M Merbromin (left) or 50 μ M HgCl₂ (right).

Figure 10. Effect of continuous exposure to γ -radiation and mercury (II) on the growth of strains containing different copy numbers of the *mer* operon. 1 x 10⁵ cells of each of the *D. radiodurans* strains R1 (*recA*⁺, *mer*⁻), *rec30* (*recA*⁻, *mer*⁻; Daly1), MD735 (*recA*⁺, 1 x *mer*⁺/cell), MD767 (*recA*⁺, 10 x *mer*⁺/cell), MD736 (*recA*⁺, 10-20 *mer*⁺/ cell), MD737 (*recA*⁺, 150 *mer*⁺/cell), and the wildtype *E. coli* strain K12 (*recA*⁺) containing pDB7 (BL308; [24]) (20-30 x *mer*⁺/cell) were spotted onto two TGY agar plates (**A** and **B**) and two TGY agar pates containing 30 μ g/ml Merbromin (**C** and **D**). Following plate inoculation, one of these plain TGY plates (**B**) and one of the TGY plus Merbromin plates (**D**) were placed into the ¹³⁷Cs irradiator (60 Gy/hour) (Gammacell 40 Irradiation Unit, Atomic Energy of Canada Ltd.) for incubation for five days. The control plates (**A** and **C**) were incubated at the same temperature in the absence of radiation for the same time.

Figure 11. Construction and characterization of a mercury resistant and toluene metabolizing *D. radiodurans*. **A**) MD560 is a previously constructed *D. radiodurans* strain that has the *tod* genes of *Pseudomonas putida* (Kobal *et al.*, 1973), encoding toluene

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dioxygenase (TDO), cloned (Lange *et al.*, 1998) the same way as the *mer* operon in MD736 (Figure 1B). The *aphA* gene (Km^R) in MD560 was replaced with the chloramphenicol resistance gene *cat* (Cm^R) forming strain MD744. This was achieved by transformation of *Xba*I (X) linearized pMD183 (Daly *et al.*, 1994b) into MD560 with Chloramphenicol (Cm) selection. MD744 genomic DNA was then transformed into strain MD737 with double Cm and Km selection, giving strain MD764. Abbreviations and symbols are as is Figure 1. **B)** Southern blotting of genomic DNA from MD764 using both a *merA*- and a *tod*-specific radiolabeled probe. E, *Eco*RI; B, *Bam*HI; λ /H, lambda phage DNA cut with *Hind*III. **C)** 2×10^5 cells of R1 (*recA*⁺, *mer*⁻), MD737 (*recA*⁺, *mer*⁺), MD764 (*recA*⁺, *mer*⁺, *tod*⁺) were spotted onto a TGY agar plate containing 30 μ g/ml Merbromin and grown in the irradiator (Gammacell 40 Irradiation Unit, Atomic Energy of Canada Ltd.) as described in Figure 4. **D)** The production of *cis*-toluene dihydrodiol (Kobal *et al.*, 1973) from toluene by strain MD764. 1×10^7 cells of strain MD764 (*mer*⁺, *tod*⁺), pre-grown in the presence of 50 μ M Merbromin, were inoculated into fresh growth medium containing 50 μ M Merbromin. Toluene was introduced in the vapor phase and the cells were incubated at room temperature with shaking at 200 rpm (Gibson *et al.*, 1970). Accumulation of metabolites in the culture medium was periodically monitored by taking a one milliliter sample, removing the cells by centrifugation, and recording the UV spectra of the diluted supernatant (1:19). UV spectra of the supernatant solutions were obtained with a Beckman DU640 spectrophotometer. Strain R1 (control) was treated in an identical manner except that it was grown in the absence of Merbromin. In a separate experiment, toluene was introduced into the vapor phase of exponentially growing cells of strains MD764 (in the presence of 50 μ M Merbromin) and R1 (in the absence of Merbromin). Two milliliter samples were periodically taken, the cells were removed by centrifugation, and the supernatants extracted twice with equal volumes of ethyl acetate. The organic extracts were dried over anhydrous sodium sulfate and evaporated to dryness. Each residue was re-dissolved in methylene chloride for analysis by thin layer chromatography (TLC) on silica using methylene chloride:ethyl acetate (1:1) as the solvent. The metabolites were located on the TLC plate by reacting with iodine vapor.

Inset: Lane 1: *cis*-toluene dihydrodiol (Fluka Chemical); Lane 2: organic extract of strain

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MD764 supernatant (20 hours); Lane 3: organic extract of strain MD764 supernatant (40 hours); Lane 4: organic extract of strain R1 supernatant (20 hours).

Figure 12. A) Mercuric Reductase Assay. Hg (II)-dependent NADPH oxidation catalyzed by cell extracts prepared from strains R1 (*mer⁻, tod⁻*; wildtype), MD735 (*mer⁺*), MD767 (*mer⁺*), MD764 (*mer⁺, tod⁺*), MD736 (*mer⁺*), and MD737 (*mer⁺*) were monitored spectrophotometrically according to the method of Fox and Walsh (Schottel *et al.*, 1978). The protein fractions (0.2 mg) were pre-incubated with 2 μ M FAD in sodium phosphate buffer containing 2-mercaptoethanol and NADPH for ten minutes, before initiating the reaction with 0.1 mM HgCl₂. Decreasing Absorbance at 340 nm corresponds to a decreasing NADPH concentration. B) Purification of mercuric reductase. Cell extracts (30 mg protein) from strains R1, MD767, MD735, MD736, MD737 and MD764 were purified for mercuric reductase as described previously using Orange A Matrex gel chromatography (Schottel *et al.*, 1978). The protein fractions eluting with NADPH were analyzed by SDS-PAGE using an 8-25% gradient PhastGel (Pharmacia Biotech AB). Lane 1: Low Molecular Weight Range Sigmamarkers (left arrows); Lane 2, R1; Lane 3, MD767; Lane 4, MD735; Lane 5, MD736; Lane 6, MD737; Lane 7, MD764; Lane 8, low molecular weight range Sigmamarkers. Size estimates: 62 kDa; 54 kDa (right arrows). C) Mercury volatilization by engineered *D. radiodurans*. Strains MD735 (*mer⁺*), MD767 (*mer⁺*), MD736 (*mer⁺*), MD737 (*mer⁺*), MD764 (*mer⁺, tod⁺*), and BL308 (*E. coli, mer⁺*) were pre-grown to 0.5 OD₆₀₀ in the presence of 20 μ M Merbromin, and also in 20 μ M HgCl₂. The control strains R1 (wildtype), MD744 (*mer⁻, tod⁺*), MD560 (*mer⁻, tod⁺*) were pre-grown to the same OD₆₀₀, but in the absence of Hg (II). Cells of each strain were harvested, washed twice in fresh medium lacking Hg (II), concentrated to OD₆₀₀ 2.0 in fresh medium, followed by the inoculation of 1 x 10⁷ cells (~50 μ l) of each into 200 μ l of medium containing 30 μ M HgCl₂ (top), or 30 μ M Merbromin (bottom), contained in 300 μ l wells of a microplate. Cells, pre-grown in Merbromin, were tested for Hg-volatilization in Merbromin-containing wells. Cells, pre-grown in HgCl₂ were tested for Hg-volatilization in HgCl₂-containing wells. Then, the plate was covered with a sheet of X-ray film, held together with a weight, and incubated in the dark at 32°C. Following

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exposure for 14 hours, the film was developed.

Figure 13. Cold vapor atomic fluorescence spectroscopy (CVAFS). *D. radiodurans* strain MD764 was grown in TGY containing Cm plus 10 μ M HgCl₂. Exponentially growing cells (150 ml; OD₆₀₀ 0.6) were harvested by centrifugation and resuspended in 75 ml TGY medium containing Cm and Km, before incubation at room temperature for two hours. The cells were harvested again, washed with TGY before being resuspended in TGY to an OD₆₀₀ of 1.8. The reaction with Hg (II) was begun by adding 25 μ l of 10 mM HgCl₂ to 25 ml of the concentrated culture (final concentration 10 μ M HgCl₂). Wildtype *D. radiodurans* strain R1 cells were treated identically except that the cells were pre-grown in TGY lacking Hg (II). At the times indicated, one milliliter samples were taken and added to 10 ml bromine monochloride and treated in a manner similar to that reported by Bloom and Crecelius (Bloome *et al.*, 1983). Appropriately diluted samples were reduced with stannous chloride and the resulting Hg (0) concentrated on gold-coated sand traps and analyzed by CVAFS using a Brooks-Rand Model III analyzer equipped with Mercury Guru 2.0 software. All solutions, including TGY, were prepared with ultra-pure water (Millipore Milli-Q Water System) and all laboratory glass- and plastic-ware were washed in warm 6 M HCl for at least twelve hours before use.

Figure 14. Transformation of *D. geothermalis* with an autonomously replication 26 kilobase plasmid (pMD66) designed for *D. radiodurans*.

20 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

I. General Description

The most radiation resistant organism yet discovered is *Deinococcus radiodurans* (Brooks *et al.*, 1980; Minton, 1996; Daly *et al.*, 1994a). *D. radiodurans* is a non-pathogenic, desiccation resistant (Mattimore *et al.*, 1996), solvent tolerant (Lange *et al.*, 1998), soil bacterium that can survive acute exposures to ionizing radiation of 15,000 Gy without lethality or increasing mutation frequency (Daly *et al.*, 1994a); this dose induces >130 double strand breaks (DSBs) per haploid chromosome (Daly *et al.*, 1994a).

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Further, this bacterium can grow continuously in the presence of 60 Gy/h (a dose rate that exceeds those at radioactive DOE waste sites [Riley *et al.*, 1992]) with no effect on either its growth rate or ability to express foreign genes (Lange *et al.*, 1998). This ability is extraordinary since most cells cannot survive more than 50-500 Gy (Thornley, 1963), or 1-
5 3 DSBs per haploid chromosome (Krasin *et al.*, 1977). Recent advances in the ability to genetically manipulate this bacterium (Lange *et al.*, 1998, Daly *et al.*, 1994b; 1995; 1996; 1997) have led to insights into its DNA repair capabilities. The mechanism of radiation resistance has been shown to be due, in part, to exceedingly efficient *recA*-dependent (Daly *et al.*, 1994a; 1994b; 1995; 1997) as well as *recA*-independent DNA repair processes
10 (Daly *et al.*, 1996). Likewise, *D. radiodurans* is also extraordinarily resistant to most chemical DNA damaging agents such as mitomycin-C, nitrous acid, and 4-nitroquinoline-*N*-oxide (Minton, 1996; Moseley *et al.*, 1983; Minton, 1994).

The ability of a microorganism to resist the toxic effect of metals is frequently associated with its ability to transform those metals to less toxic chemical states. Cloning
15 metal resistance genes into *D. radiodurans*, therefore, serves two important objectives: 1) to confer resistance to the most common metallic waste constituents; and 2) to transform those metals to less toxic and less soluble chemical forms. Generally, the solubility of metals is reduced at lower oxidation states, and enzymes catalyzing such metal reducing functions are becoming important components of metal bioremediation strategies. For
20 example, the bacterial mercuric reductase gene, *merA*, encodes mercuric ion reductase (MerA), that reduces highly toxic, thiol-reactive mercuric ion, Hg (II), to much less toxic and nearly inert monoatomic Hg (0) (Hamlett *et al.*, 1992). Ionic Hg (II) is a frequent metal contaminant at DOE facilities (Riley *et al.*, 1992; McCullough *et al.*, 1999); there may be as many as 250 DOE waste sites contaminated with Hg (II) (Riley *et al.*, 1992).
25 Mercuric (II) ions are extremely toxic to humans and other organisms due to their avid binding to sulfhydryl groups and, therefore, inhibit many enzyme-catalyzed reactions (Creighton, 1993). While some bacteria activate Hg (II) to more toxic forms (*e.g.*, dimethylmercury), others can detoxify and remediate the ion via a reductive enzymatic reaction that produces volatile elemental mercury. Mercury (0) is relatively non-toxic to
30 bacteria, plants, animals, and humans. The genes responsible for the reaction, most

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notably the gene mercuric reductase (*merA*), are widely distributed in bacteria, and have been cloned and expressed in transgenic plants (Rugh *et al.*, 1998). MerA is a member of the flavoprotein redox-active disulfide family of proteins.

To demonstrate the applicability of the strategy to confer both metal resistance and metal remediating capabilities on a radiation resistant bacterium, the present inventors cloned the highly characterized *merA* locus from the *Escherichia coli* strain BL308 (*E. coli* K12 containing pDB7, [Barineau *et al.*, 1984]) into *D. radiodurans* (Rainey *et al.*, 1997; White, 1999). Four different *D. radiodurans* expression systems were tested. Further, by designing bioremediating *D. radiodurans* targeted at specific, and possibly unique, radioactive sites, the present invention includes bacterial strains engineered to combine a variety of different gene-encoded functions into a single host.

One embodiment of the invention includes extremely radiation resistant *D. radiodurans* strains which express *mer*-encoded gene functions and are: 1) resistant to the bacteriocidal effects of ionic Hg (II) at concentrations (50 μ M; Figure 9D) well above the highest concentration reported for mercury-contaminated DOE waste sites (10 μ M [Riley *et al.*, 1992]); and 2) reduce toxic Hg (II) to much less toxic elemental and volatile Hg (0) (Figures 12, 13).

II. Specific Embodiments

Organic Toxin Degradation

While microorganisms can degrade most natural compounds, few are able to degrade synthetic compounds such as fuel hydrocarbons (*e.g.*, toluene) or halogenated hydrocarbons (*e.g.*, TCE or PCBs). The scarcity of appropriate microbial enzyme systems to degrade these serious recalcitrant hazardous pollutants (Infante *et al.*, 1982, Jacobson *et al.*, 1996) is a reflection of the relatively recent introduction of these xenobiotics to Earth. Since the introduction of these compounds into the biosphere about fifty years ago, natural evolution has begun to modify pre-existing bacterial genes to make enzymes capable of metabolizing such synthetic organic chemicals (Chakrabarty, 1996). In the last fifteen years, researchers have identified some of these genes (mostly from *Pseudomonas* spp.)

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and have begun to refine their pollutant-degrading capabilities by genetic engineering (Wackett, 1997, Wackett *et al.*, 1988).

The use of *in situ* bioremediation for organic toxin-contaminated soils and groundwaters poses as a viable alternative to chemical methods that utilize costly pump and treat technologies (McKay *et al.*, 1989) and/or soil excavation and incineration. Typical organic solvents used by DOE: benzene, toluene, ethylbenzene and xylenes (collectively called BTEX), are known growth substrates for some organisms (*e.g.*, *Pseudomonas* spp.), of which the genetics and biochemistry have been studied in great detail. Furthermore, it has been discovered that co-contaminating haloorganic solvents such as TCE are biotransformed (co-oxidized) during aerobic metabolism of certain aromatic compounds (*e.g.*, toluene) where broad specificity oxygenases from toluene catabolic pathways can, typically, co-oxidize TCE. Compounds such as high molecular weight PCBs, that were originally thought to be non-degradable by microbes, are regularly being found to be transformed by bacteria utilizing biphenyl and low molecular weight PCBs for growth (Focht, 1995). With respect to DOE facilities, up until now, there has been no adequate method for microbiological treatment of contaminant waste sites containing both hazardous organic and radioactive components since organisms like *Pseudomonas* spp. are very radiation sensitive.

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Table 1. Distribution of Compound Classes in Soil⁵ and Groundwaters and the Number of Sites (out of 91) Containing Mixtures of those Compounds as a Function of DOE Facility¹.

		Distribution (by % of waste Sites) ² of Compound Classes ³				Number of Reported Compound Class Combinations				
Facility	#	A ⁴	B ⁴	C ⁴	D ⁴	A+B ⁴	A+C ⁴	A+D ⁴	A+B+C ⁴	A+B+D ⁴
Argonne Nat. Lab.	2	0 10 0	0 50 0	0 0 0	0 0 0	1				
Brookhaven Nat. Lab.	4	0 25 0	0 10 0	0 0 0	0 75 0	1				
Fernald	11	72 72 0	45 72 0	10 0 0	27 72 0	3 8 0	8	1 8 0	3	1
Hanford Site	7	85 71 0	28 42 0	14 0 0	14 0 0	2 3 0	1	1	1	
Idaho Nat. Engin. Lab	6	50 50 0	33 66 0	33 0 0	16 16 0	2 2 0	2	1 1 0	2	1 1 0
Oakridge Nat. Lab.	9	33 44 0	11 77 0	33 0 0	22 22 0	1 3 0	2	1 2 0	1	1 2 0
Pantex Plant	3	0 66 0	66 33 0	0 0 0	10 0 33	1		1		
Rocky Flats Plant	3	66 10 0	10 0 10 0	0 0 0	33 33 0	2 3 0		1 1 0		1 1 0
Savannah River Plant	9	33 77 0	55 77 0	0 0 0	33 0 0	1 6 0				

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¹ Data obtained from Riley *et al.*

: Number of sample waste sites evaluated at a specific facility.

² Percent of sampled waste sites at a specific facility reporting a specific compound class.

³ Compound-class index:

5 A = **radionuclides and toxic metals**

 B = **chlorinated hydrocarbons**

 C = **polychlorinated biphenyls**

 D = **fuel hydrocarbons**

10 ⁴ Column cell numbers refer to compound class in soil (top) and in groundwater (bottom
italic).

⁵ Soils and Sediments.

Many subsurface environments are anoxic and anaerobic microorganisms are being isolated from these environments that are capable of transforming pollutants. For example, toluene and benzene are known to be degraded anaerobically using alternative
15 electron acceptors other than O₂ and TCE is dehalogenated by certain anaerobes to less halogenated ethylenes. Examples of these anaerobic bacteria include dissimilatory iron-reducing bacteria (DIRB) that can utilize ferric iron associated with aqueous or solid phases as a terminal electron acceptor coupled to the oxidation of organic substrates (Lovley, 1991). When stimulated for Fe(III) reduction, such microbes can efficiently
20 remove hydrocarbons (*e.g.*, benzene) in anoxic environments. However, the genetics and biochemistry of anaerobic processes are, in general, poorly characterized and many of the genes encoding these activities are not yet identified/cloned (Lovley, 1995).

Even when the dissolved O₂ concentrations of groundwaters are between 0-400 μM, aerobic systems can be generally applied to contaminated groundwaters. In those
25 environments where oxygen is limiting, bioventing and biosparging strategies have been developed to circumvent this problem. The general rule is that if there is any oxygen present, it will likely be the primary terminal electron acceptor, and aerobic processes will function, although perhaps slower at low O₂ concentrations. The use of biodegradation pathways from aerobes, such as those described herein, is not of primary concern because

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the typical K_m for O_2 by those enzymes employed in such pathways is much lower than the concentrations of O_2 typically found in environments with low oxygen tensions, such as in groundwater.

Metal Toxicity Resistance

5 A number of biological systems have been described for resistance to heavy metals which may be used to increase the resistance of *D. radiodurans* to the toxic effects of metals and radionuclides present in DOE wastes (Riley, 1992). If *D. radiodurans* is not naturally resistant to metals over the concentration ranges that are found at DOE sites, strains of the invention can be engineered for resistance to those metals by either natural
10 selection or by genetic engineering. For instance, many heavy metal resistance (export) systems are functional in *Alcaligenes eutrophus* CH34 (Diels *et al.*, 1995) that has multiple heavy metal resistance genes, and many modes of detoxification. These genes include: *czc* (Cd^{2+} , Zn^{2+} , Co^{2+}), *cnr* (Co^{2+} , Ni^{2+} , Zn^{2+}), and *mer* (Hg^{2+} and organomercury), as well as other genes recently cloned for resistance to Cu^{2+} , Pb^{2+} and Mn^{2+} , all of which
15 may be cloned and expressed in *Deinococcus* as described below.

An alternative to using *A. eutrophus*' metal-exporting genes, is cloning metallothionein (MT) genes into *Deinococcus*. The Cyanobacterium *Synechococcus* produces MT-like proteins that provide resistance to the toxic effects of Zn^{2+} , Cd^{2+} , and Hg^{2+} by intracellular sequestration. In *E. coli*, expression of the *Synechococcus* genes
20 encoding the MT-like proteins caused enhanced intracellular accumulation of Zn^{2+} , Cd^{2+} and Hg^{2+} . MT-like proteins have also been isolated from Cd^{2+} -resistant *Pseudomonas putida* (Higham *et al.*, 1984). The cloned genes encoding these low molecular weight MT-like polypeptides may be introduced into any of the *Deinococcal* or other radiation resistant strains, including those described herein. For resistance to the semi-metal
25 arsenic, cloning the *arsB* and *arsC* As-efflux resistance genes from the Gram-positive genus *Staphylococcus* are also expressible in *Deinococcus*. The metals found most frequently associated with radionuclides at DOE sites are listed in Table 2 (the highest groundwater concentrations in 'mM' are also shown).

Table 2

Metal	Groundwater Concentration (µg/L)	Ground Concentration (µg/kg)
Lead	0.56 - 120,000 (1.1 mM)	1,000 - 6,900,000
Chromium	0.42 - 9,010 (0.17 mM)	5.1 - 3,950,000
Arsenic (semi-metal)	0.3 - 32,100 (0.4 mM)	100 - 102,000
Zinc	1 - 697,000 (10.7 mM)	150 - 5,000,000
Copper	1 - 3,300 (0.052 mM)	30 - 550,000
Cadmium	0.005 - 7,600 (0.06 mM)	100 - 345,000
Mercury	0.08 - 216,900 (1.1 mM)	0.1 - 1,800,000

DOE Wastes Targeted for Bioremediation Research

The individual organic chemical constituents of radioactive wastes sites targeted for microbiological remediation are given in Table 3. On the basis of the frequency of occurrence, the following organic chemical representatives of specific compound classes may be the primary targets for the bioremediation bacteria, compositions and methods of the invention: 1) fuel hydrocarbon class: toluene; 2) chlorinated hydrocarbon class: trichloroethylene; 3) PCB class: Arochlor 1248.

Table 3. Among the 91 DOE Sites^A Screened, Number of Selected Compound Class Constituents in Soils^B and Groundwaters.

5

Compound Class	Class constituent	Ground ^C	Groundwaters ^C
Chlorinated hydrocarbons	Trichloroethylene	11	14
	1,1,1 -Trichloroethane	10	11
	Tetrachloroethylene	9	10
	Dichloroethane	9	7
	Carbon tetrachloride	6	7
	Chloroform	5	10
Fuel hydrocarbons	Toluene	8	8
	Xylene	5	8
	Ethylbenzene	5	6
	Phenanthrene	4	NR ^D
	Anthracene	4	NR ^D
PCBs	Arochlor 1248	4	0
	Arochlor 1016	1	1
	Arochlor 1242	2	2
Radionuclide/<u>Metal</u>	Uranium (γ , α) ^E / <u>Lead</u>	12 / <u>16</u>	12 / <u>5</u>
	Plutonium(α) ^E / <u>Chromium</u>	10 / <u>13</u>	5 / <u>6</u>
	Cesium (γ , β) ^E / <u>Arsenic</u>	10 / <u>13</u>	5 / <u>6</u>
	Tritium (β) ^E / <u>Zinc</u>	6 / <u>13</u>	12 / <u>6</u>
	Strontium (β) ^E / <u>Copper</u>	6 / <u>12</u>	9 / <u>7</u>
	Thorium (α) ^E / <u>Cadmium</u>	3 / <u>10</u>	5 / <u>6</u>

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^A Data obtained from ref. 81.

^B Soils and Sediments.

^C Number of sample waste sites (out of 91) reported at 18 DOE Facilities.

^D Not Reported.

5 ^E Mode of decay.

Recombinant Bacterial Strains

The recombinant bacteria of the invention are resistant to the damaging effects of radiation. Preferred strains are resistant to acute exposure to ionizing radiation of up to about 15,000 Gy or are resistant to continuous or chronic exposure to ionizing radiation of
10 up to about 60 Gy/hour. Bacterial species that may be engineered include species and strains of *Enterococcus*, *Alcaligenes* and *Deinococcus*, although other radiation resistant bacterial species or species of other microorganisms may be used. Radiation resistant strains of *Deinococcus* include, but are not limited to, strains of *D. radiodurans*, *D. radiopugnans*, *D. grandis*, *D. proteolyticus*, *D. murrayi*, *D. geothermalis*, and *D.*
15 *radiophilus* as well as other uncharacterized *Deinococcus* isolates. Preferred bacterial strains are engineered strains of *D. radiodurans* and *D. geothermalis*.

Bacterial strains of the invention may be engineered using any available technologies, including available plasmids or vectors, selection markers, transformation systems or methods, etc. For instance, a number of autonomously replicating plasmids
20 and chromosomal integration vectors for *D. radiodurans* are available that allow the expression of heterologous genes at high copy number (Daly et al., 1994a; 1994b; 1995; 1996; 1997)

There are at least four expression systems available for *D. radiodurans* that are summarized below. For example, the present inventors have developed a large number of
25 shuttle vectors including integrating and plasmid vectors for use in *D. radiodurans* and *E. coli*. These vectors are used to express foreign genes in *D. radiodurans* and *D. radiodurans* genes in *E. coli*. The first *D. radiodurans* plasmids, typically, were composed of an *E. coli* plasmid containing a kanamycin resistance gene (Km^R) and some *D. radiodurans* chromosomal DNA (Smith et al., 1988). In *E. coli* these plasmids replicated

autonomously, but in *D. radiodurans* they conferred Km^R by chromosomal insertion. This type of expression is summarized below as a Type-IV expression system.

The *D. radiodurans* expression vectors typically consist of an assortment of characterized DNA segments containing discrete functional elements (*e.g.*, for promoting or replicating). One class contains autonomously replicating plasmids, while the remaining three contain chromosomal integration vectors. All four vector types can be propagated in *E. coli* and subsequently used to transform *D. radiodurans* and other *Deinococcal* strains, such as *D. geothermalis*. These specialized *Deinococcus-E. coli* plasmids were tailored for optimal function and are highly characterized (Daly *et al.*, 1994a; 1994b; 1995; 1996; Smith *et al.*, 1988). Generally, expression of cloned genes in *D. radiodurans* is regulated by varying foreign gene dosage in combination with constitutive promoters.

Type-I: Autonomous plasmids: These plasmids are autonomously replicating DNA circles in *D. radiodurans* and, typically, contain two constitutive promoters; one for driving an antibiotic resistance gene, the other for driving a foreign gene. They exist at six copies per cell (Daly *et al.*, 1994a).

Type-II: Chromosomal direct-insertion vectors: Vectors of this class will integrate into the *D. radiodurans* chromosome by homologous recombination, leaving a single permanent copy per chromosome (there are 4-10 identical chromosomes per cell). Foreign genes integrated into the chromosome this way are promoted by adjacent constitutive *D. radiodurans* promoters (Daly *et al.*, 1995).

Type-III: Chromosomal duplication-insertion vectors: These vectors will integrate into the chromosome by homologous recombination leaving 10-20 transient copies per cell. Unlike a Type-II insertion, a Type-III chromosomal insertion can be lost by extended growth in the absence of any selection, restoring the original chromosomal sequence. Foreign genes located within these chromosomal insertions are expressed by a constitutive promoter (Carrol *et al.*, 1996, Daly *et al.*, 1995, Daly *et al.*, 1996).

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Type-IV: Chromosomal amplification vectors: These vectors are very similar to Type-III vectors. However, upon homologous integration, these vectors amplify in the chromosome yielding 80-500 vector copies per cell. Expression of foreign genes, within the amplification unit, is proportional to the number of integration copies per cell (Smith *et al.*, 1988).

The four expression systems for *Deinococcus* summarized above can be combined into the same host cell. For instance, the present inventors have constructed a number of *D. radiodurans* strains, each containing two different gene expression types marked with either resistance to kanamycin (Km^R) or chloramphenicol (Cm^R) (*e.g.*, Km^R-Type-II plus Cm^R-Type-III (Daly *et al.*, 1995); Km^R-Type-III plus Cm^R-Type-III (Daly *et al.*, 1996); Km^R-Type-I plus Cm^R-Type-III (Dowling *et al.*, 1993)). These different combinations were initially selected for in the same hosts by double drug selection and were shown to be maintained by cells even without any selection following irradiation (Daly *et al.*, 1995; Daly *et al.*, 1996; Daly *et al.*, 1997). Any of these vectors may be used to engineer bacterial strains of the invention. Preferred strains may express metal resistance genes using Type-II (direct-insertion) vectors and toxin-degrading genes using Type-III and Type-IV chromosomal insertion using vectors.

Engineered bacterial strains of the invention may contain any available genes, loci or operons that encode proteins that degrade, metabolize or detoxify toxins such as organic chemicals, metals or other compounds found in waste sites. For instance, the *P. putida* *todC1C2BA* and *E. coli* *merA* operons may be cloned into the radiation resistant bacterial strain of choice. These genes may be used to augment the native ability of the recombinant strains to degrade or detoxify toxins or heavy metals. Numerous other degradatory or resistance functions from other bacteria, such as resistance functions specific for metals, may be cloned as set forth in Table 5.

Table 5 Degradative Pathway Genes or Metal Resistance Genes

	Substrate	Degradative Pathway Genes / Plasmid or Metal Resistance Genes / Plasmid	Organism	Reference
	toluene/TCE	<i>todC1C2BA</i> / pHG-2 cloned into <i>D. radiodurans</i>	<i>Pseudomonas</i>	111
	toluene/TCE	Tol region / pDKR1; pRP1	<i>Pseudomonas</i>	113
5	toluene	<i>xylL-xylE</i> / pBK187	<i>Pseudomonas</i>	107
	toluene	upper and lower TOL operons / pWW53-4, pEHK11, pEHK355	<i>Pseudomonas</i>	53
	toluene	monooxygenases: / pMS64, PK01, KR1	<i>Pseudomonas</i>	52,73, 80, 89
	TCE	gene clusters involving <i>bphA1A2A3A4</i> and <i>todC1C2BA</i> / pJHF3051, pJHF301 and pJHF108	<i>Pseudomonas</i>	32
	PCBs	<i>bph</i> gene operon subclones/ pDD5301, pDD530, pDD5201, pDD5211	<i>Pseudomonas</i>	2, 8, 21
10	Cd, Zn, Co, Hg, Mn, Pb	<i>czcC, czcB, czcA</i> pMOL30	<i>Alcaligenes eutrophus</i> , <i>Ralstonia eutrohus</i>	20
	Zn, Cd, Hg	<i>smtA, smtB</i>	<i>Synechococcus</i>	102
	As (semi-metal)	<i>arsA, arsB</i>	<i>Staphylococcus</i>	50, 86
15	U(VI)	<i>cytc3</i>	<i>Desulfovibrio vulgaris</i>	
	Cr(VI)		<i>Bacillus thuringiensis</i>	

Growth Media

The present inventors have also developed a synthetic minimal media which may be used to engineer strains of the present invention and to practice the claimed methods.

- 20 In developing a synthetic minimal medium, many combinations of varying amounts of carbohydrates, amino acids, salts and vitamins in both liquid and solid medium were systematically tested. By a process of elimination, minimal nutrient constituents, and their concentrations, necessary for luxuriant growth were identified as set forth in Table 5. This synthetic medium preparation is distinct in that it is much simpler, and growth of *D.*

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radiodurans in such medium is completely dependent on a carbon/energy source. In addition to a metabolizable carbon source, growth of *D. radiodurans* is dependent on exogenous amino acids and a vitamin; addition of the sulfur-rich amino acids cysteine and histidine, together with nicotinamide were particularly effective at supporting growth.

- 5 However, the specificity of amino acids was shown is not stringent in that many different combinations of amino acids support growth. A factor that strongly influences the extent of growth is the total amino acid concentration in the growth medium, and not the composition of the amino acid pool. Among the carbon sources tested, the following supported luxuriant to slow growth in the following order: fructose > pyruvate > lactate >
- 10 glucose > oxaloacetate > acetate > glycerol.

Table 5. *D. radiodurans* minimal nutrient requirements for growth in the absence (A) and presence (B) of γ -radiation

		<u>Compounds:</u>		<u>Concentrations:</u>	
				A	B
15	BSM:	Potassium Phosphate Buffer (pH7.5-8.0)		20 mM	20 mM
	Salts:	Magnesium Chloride, Tetrahydrate		0.2 mM	0.2 mM
		Calcium Chloride, Dihydrate		0.1 mM	0.1 mM
		Manganese(II) Acetate, Tetrahydrate		5.0 μ M	5.0 μ M
20		Ammonium Molybdate, Tetrahydrate		5.0 μ M	5.0 μ M
		Ferrous Sulfate, Heptahydrate		5.0 μ M	5.0 μ M
Amino Acids:					
		Histidine		25 μ g/ml	2.5 mg/ml
		Cysteine		25 μ g/ml	2.5 mg/ml
25	Vitamin:	Nicotinic Acid		1.0 μ g/ml	1.0 μ g/ml
	Carbon:	Carbon Source		2 mg/ml	2mg/ml

Basal Salt Medium (BSM) was autoclaved and then supplemented with sterile

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preparations of salts, amino acids and nicotinamide, to the indicated concentrations. For solid medium, Nobel Bacto Agar was added before autoclaving BSM, to 1.5% (w/v). Individual carbon sources were added to a concentration of no more than 2 mg/ml. The concentrations shown on the left (**A**) are those used for growth in the absence of radiation.

- 5 The concentrations shown in bold on the right (**B**) are changes made to nutrient conditions shown in A that supported growth in the presence of continuous radiation (60 Gy/hour). Growth media for continuous radiation exposure may also be supplemented with other amino acids at the following approximate concentrations: glutamine, 500 µg/ml; alanine, 500 µg/ml; arginine, 800 µg/ml; asparagine, 800 µg/ml; glycine 300 µg/ml; leucine, 500
10 µg/ml; lysine, 300 µg/ml; methionine, 100 µg/ml; proline, 370 µg/ml; serine 300 µg/ml; threonine, 200 µg/ml; tryptophan, 200 µg/ml; tyrosine, 200 µg/ml; and valine, 200 µg/ml. Substitution of Nicotinic acid with Basal Medium Eagle Vitamin Solution (GibcoBRL) improves growth slightly.

Contaminated Waste

- 15 As used herein, the term "toxins" includes organic, radionuclide and inorganic or metallic contaminants, as well as non-petroleum organic contaminants, particularly those found in industrial waste, waste generated from the production of nuclear weapons and waste produced from the civilian uses of radionuclides. Such contaminants are also often found in waste generated from textile and paper mills, chemical manufacturers, and
20 transportation facilities, as well as restaurants and institutions, such as commercial kitchens, food processing plants, and the like. Other sources of contaminant production include crude oil spills, chemical and solvent leaks, fuel oil leaks, and creosote contamination.

- As used herein, inorganic contaminants include the contaminants described above
25 as well as inorganic sulfur and ferrous compounds, metallic elements, such chromium, lead, arsenic, zinc, cadmium, cobalt, mercury and certain copper compounds used as herbicides and algicides.

- Organic contaminants include the contaminants described above as well as various pesticides, such as insecticides, growth regulators, growth inhibitors, toxicants,
30 bactericides, attractants, repellants, hormones, molluscicides, defoliants, chemosterilants, fumigants, systemics, rodenticides, avicides, detergents, surfactants, nematicides,

acaricides, miticides, predicides, herbicides, agricultural chemicals, algicides, fungicides, sterilants; polycyclic aromatic hydrocarbons (PAH's), polychlorinated biphenyls (PCB's), greasy wastes, solvents, crude oil, diesel fuel, waste oil, Bunker "C" oil, phenolics, halogenated hydrocarbons, citrus juice processing wastes, terpene alcohols, starchy carbohydrates, and the like.

Examples of specific organic contaminants include those described above as well as anthracene, chlorotoluenes, chrysene, cresols, di-N-octylphthalate, dichlorobenzene, dichlorethanes, dichloropropanes, dichlorotoluene, 2-ethoxyethanol, ethylene glycol, ethylene glycol monoethyl ether acetate, ethylbenzene, fluorene, isoprenoids, methyl ethyl ketone, methylene chloride, naphthalene, pentachlorophenol, phenanthrene, 1,1,2,2-tetrachloroethane, toluene, 1,1,2-trichloroethane, trichloroethylene, benzoate, chlorobenzoates, methanol, ethyl acetate, cyclohexanone, ethylbenzene, 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, m,o,p-xylene, butyl acetate, camphor, hexane, heptane, octane, nonane, d-limonene, linalool, geraniol and citronellol.

Bioremediation compositions

Bioremediation compositions of the invention may be engineered and formulated to meet the applicable regulatory requirements, including the requirements of the Department of Energy as outlined in McCullough *et al.*, Bioremediation of Metals and Radionuclides, which is herein incorporated by reference in its entirety. For instance, bioremediation compositions may include film-forming agents and/or nutrient agents in additions to the bacterial strains of the invention. These can be used singly or in various combinations. In a preferred embodiment for use in aqueous environments or clean-up sites, bioremediation compositions may comprise at least one film-forming agent. The use of a film-forming agent in combination with a bacterial strain of the invention generally acts to enhance the activity of the bacterial strain. For example, film-forming agents can be used to increase the surface area where oil is a major contaminant by uniformly spreading a bacterial strain of the invention throughout a thin layer of dispersed oil, thereby accelerating the biodegradation process. Not only is the oil made more readily

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available to the bacterial strain, but mixing and dilution of emulsion droplets in a greater volume of water assures a more adequate supply of nutrients for the bacterial strain utilized.

Film-Forming Agents

- 5 As used herein, the term "film-forming agent" is meant to include dispersants, surface-active agents, surfactants, detergents, and the like. Typically, this class of chemicals have an oil-soluble end (*i.e.*, a hydrocarbon chain) and a water soluble end (*i.e.*, polar groups, such as carboxylate, sulfonate, ether, alcohol, or polyethylene oxide). Because of this dual nature, film-forming agents orient at the surface contaminant/water
- 10 interface. Surface contaminants refer to contaminants which have a tendency to float on the surface of water. Specific examples of surface contaminants include oil or petroleum. When applied to aqueous waste contaminated with oil, for example, a film-forming agent will reduce the surface tension of the water while spontaneously and rapidly spreading over the surface of the water to form a near monomolecular or duplex film that can push or
- 15 concentrate oil or other surface contaminants into a confined area for clean-up. Alternatively, when applied to oil, the oriented film-forming molecules can also reduce the interfacial tension between the oil and water thereby "weakening" and reducing the cohesiveness of the oil slick. Additionally, the hydrophilic groups of the film-forming molecules on the surface of the oil droplets repel other droplets and prevent coalescence.
- 20 The hydrophilic surface also reduces the tendency of the droplets to stick to solid.

Film-forming agents that are suitable for use in the present invention are generally more oil soluble than water soluble and preferably are only minimally water soluble. Furthermore, film-forming agents suitable for use in the present invention are organic materials which spread rapidly and spontaneously into extremely thin films approaching

25 monomolecular dimensions. Consequently, small quantities of film-forming agents will affect large areas of a water surface. These film-forming agents are generally autophobic, nonionic, nonvolatile organic liquids with a density less than water. Typically, they have a low freezing point and a boiling point above the maximum air temperature of the environment into which they are placed. The freezing point can be below about 5°C. The

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boiling point can be about 170°C or higher, preferably it is at least about 200°C. These film-forming agents have an HLB (Hydrophile Lipophile Balance) number of 10 or less, a bulk viscosity of less than 1000 centistokes at the temperature of use, a surface tension effectiveness which lowers the surface tension to approximately 35 dynes/cm or less, and
5 are generally capable of rapidly and spontaneously spreading with high spreading potentials.

Suitable film-forming agents include, but are not limited to, POE-2-isostearyl alcohol, sorbitan monooleate, sorbitan trioleate, sorbitan monolaurate, oxyethylated oleyl alcohol having two oxyethylene groups, diethylene glycol monolaurate, oxyethylated
10 lauryl alcohol having four oxyethylene groups, an oxyethylated branched alkanol of 15-19 carbon atoms, unsaturated cis-alkanol of 12-18 carbon atoms and up to five oxyethylene groups, and an unsaturated cis-alkanol of 15-19 carbon atoms. These film-forming agents can be used in combination with an alcohol such as 2-ethyl butanol, for example. Most preferably, the film-forming agent is selected from the group consisting of
15 POE-2-isostearyl alcohol, 65% sorbitan monolaurate and 35% 2-ethyl butanol, and 75% sorbitan monooleate and 25% 2-ethyl butanol.

Nutrient Agents

Bioremediation compositions in accordance with the invention can also include nutrient agents. As used herein, the term "nutrient agent" is defined as any substance that
20 accelerates degradation by stimulating the growth of a bacterial strain of the invention. Nutrient agents can be composed of macronutrients, micronutrients, or mixtures of both. Generally, the nutrient agents include carbon sources, nitrogen sources, phosphorous sources, or mixtures thereof. Examples of specific nutrient agents that can be used in accordance with the invention are the nutrients described in Table 4 as well as the
25 BI-CHEM ACCELERATOR series (available from Sybron Biochemicals Inc., Birmingham NJ). The nutrient agent employed will vary according to the particular bacterial strain being used to control contaminants, as well as the environmental context of its application.

One embodiment of the invention includes the use of nutrient agents to enhance the

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activity of the bacterial strain. In some instances, the nutrient agent and the bacterial strain are both impregnated within a polymer to enhance bioremedial activity.

Controlled Release Contaminant-Reducing Agent Delivery Compositions

The bioremediating compositions of the present invention can be prepared by
5 mixing, encapsulating, agglomerating, or formulating one or more bacterial strains of the invention with one or more non-toxic and inert adjuvants or diluents into compositions such as solid powders, dusts, granules, pellets, briquets, extrusions, laminates, or composites, or into sprayable, pumpable, or injectable, variable-viscosity water or oil-base formulations such as gels or semi-gels. These compositions can be optionally
10 incorporated into water-soluble or biodegradable/degradable packets, pouches, or capsules, made of, for example, polyvinyl alcohol, hydroxypropyl methyl cellulose, polyethylene oxide, or gelatin, or insoluble devices made, for example, of polyethylene or polypropylene, for use as secondary delivery vehicles for contaminant-reducing compositions.

15 In particular, the present invention is directed toward a method of formulating one or more bacterial strains of the invention, with or without water or other additives, into compositions such as solid powders, dusts, granules, agglomerates, pellets, briquets, extrusions, laminates, or composites, or into sprayable, pumpable, or injectable, variable-viscosity water or oil base gel or semi-gel like formulations that can release one
20 or more active ingredients to simultaneously or concurrently control a variety of inorganic or organic contaminants with a single or multiple application of a solid or liquid single or multi-product formulation. Preferably, the release occurs in a controlled manner.

The slow or controlled release process may be modified or delayed by the degree of compaction of the formulation, by varying the size of an orifice or the number of
25 orifices in a container into which the formulation is placed, by varying the concentration of film-forming agent, by varying the concentration of different types of polymers, and by adding one or more binders. For example, using one or more cationic, anionic, or nonionic surfactants or surface active agents in the composition can regulate the rate and duration of delivery (*i.e.*, increase or decrease).

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Preferably, the bioremediating compositions of the present invention contain one or more nontoxic and inert adjuvants or diluents such as carriers, binders, coatings, defloculating agents, penetrants, spreading agents, surface-active agents, surfactants, suspending agents, wetting agents, stabilizing agents, compatibility agents, sticking
5 agents, waxes, oils, co-solvents, coupling agents, foams, antifoaming agents, synthetic plastics, elastomers, synergists, natural or synthetic polymers, UV protectants, buoyancy modifying agents, biocides, and other additives and mixtures thereof. Some materials may be biodegradable, or photodegradable (*e.g.*, ultraviolet light), and others may be degraded by hydrolysis.

10 Bioremediating compositions of the invention can be applied to the contaminated site by conventional ground, aquatic or aerial techniques as outlined by McCullough *et al.*, 1999. In a terrestrial environment, the composition can be applied directly on the soil surface, introduced into one or more sub-surface layers, mulched into the soil, introduced into biopiles or prepared beds or composted with contaminated soil or materials. The
15 methods of the invention may also include the use of bioreactors and other bacterial growth augmentations methods.

In an aquatic environment, the composition may be applied to uniformly mix within the aquatic environment or be applied at or near the surface of water. Slurry bioreactors and sediment washing equipment may also be used in the methods of the
20 invention. When applied directly to a contaminated water source, the bioremediating composition can be applied at a total bulk application rate of about 0.1 to about 2000 pounds per surface acre of the target environment. More preferably, the bioremediating composition is applied at a total bulk application rate of about 0.1 to about 500 pounds per surface acre of the target environment. The application range will depend upon the type of
25 agent used, any polymers employed, the duration and rate of release desired, the total application rate required to uniformly treat the area of contamination, the type and concentration of contaminant, and the concentration of natural contaminant-reducing organisms and nutrients in the target habitat.

Without further description, it is believed that one of ordinary skill in the art can,
30 using the preceding description and the following illustrative examples, make and utilize

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the strains and bioremediation compositions of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

5

EXAMPLES

Methods

The following methods were used as described below:

Growth of Cells: *D. radiodurans* and *E. coli* strains were grown in TGY medium and Luria-Bertani (LB) medium, respectively, with aeration on rotary shakers at 32°C and
10 37°C, respectively. Kanamycin was used at a concentration of 8 µg/ml for recombinant *D. radiodurans* strains.

Strain Construction: The regional chromosomal maps and functions of *D. radiodurans* strains MD417 and MD560 are shown in Figure 1. The tandem duplication vector pMD417 and *D. radiodurans* control strain MD417 (lacking *tod* genes) were
15 constructed and described previously. Strain MD560 is identical to strain MD417 except for the presence of the *todC1C2BA* genes. An *EcoRI-BamHI* (4.2 kb) fragment containing the *todC1C2BA* genes (Zylstra *et al.*, 1989) was cloned from plasmid pHG2 (Wackett *et al.*, 1994) into pMD417 (Figure 1) forming plasmid pMD532. MD560 is the product of transformation of wildtype strain R1 with pMD532 followed by selection on TGY plates
20 containing kanamycin (Results). pMD532 cannot replicate as a plasmid in *D. radiodurans* because of the absence of a deinococcal plasmid origin of replication. Upon transformation, integration of pMD532 into the chromosomal target sequence BC (checkered segments, Figure 1) occurs by homologous recombination (a single cross-over) between the BC regions of the plasmid and the chromosome, respectively. As a result, the
25 integrated vector becomes flanked on both sides by chromosomal BC sequences, forming a chromosomal tandem duplication. In *D. radiodurans*, chromosomal region 560 can confer Km^R (resistance to kanamycin encoded by a portion of the *E. coli* plasmid pMK20 [diagonally hatched region, Figure 1] that contains the *aphA* gene). Transcription of the *aphA* gene is driven by *Deinococcal* constitutive promoting sequences in a fragment

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derived from the *D. radiodurans* SARK natural plasmid pUE11 (black region, Figure 1). Transcription of the TDO genes in strain MD560 are driven by deinococcal constitutive promoting sequences in a fragment derived from the *D. radiodurans* SARK natural plasmid pUE10⁵ (light grey region, Figure 1).

5 *Analysis of Substrate Degradation:* Toluene, chlorobenzene, 3,4-dichloro-1-butene, and TCE degradation studies were measured initially in eleven milliliter sealed reaction vials with one milliliter of concentrated cells at about 1×10^9 cells/ml and 25 nmol of substrate. At timed points over a one hour period, 50 ml of headspace was removed from reaction mixtures with a gas tight syringe and analyzed on a
10 Hewlett Packard 5890 GC with flame ionization detector using a DB-1 capillary column (0.25 mm ID, 0.25 mM film thickness, 30 m length) operating at an isothermal oven temperature of 180°C, splitless injection at a temperature of 250 °C, and peak integration. The toluene and chlorobenzene *cis*-dihydrodiols were extracted from culture supernatants with ethyl acetate and analyzed by thin layer chromatography with ethyl acetate as solvent
15 and by gas chromatography-mass spectrometry analysis using a Hewlett Packard 6890 GC with mass selective detector and Chemstation. All data were consistent with previous reported values. Further, identical products were formed with MD560 cell incubations using unconcentrated cells (OD_{600} 0.8-1.2) over a period of 12 hours (data not shown). ¹⁴C-TCE experiments were conducted in sealed eleven milliliter vials using strains
20 MD560, MD417, and a TGY control, to which 1 μ Ci, 20 μ l of ¹⁴C-TCE (8.5 mM in DMF, specific activity 6 μ Ci/mmol), was added each to one milliliter of cells at a density of 1×10^8 cells/ml. A zero time point and 18 hour time point were taken by removing 20 μ l of mixture and applying the 20 μ l to a 1 cm x 1 cm silica TLC plate to dry. After air drying, the TLC plates were added to five milliliter scintillation cocktail and residual
25 nonvolatile ¹⁴C measured.

For detection of indole oxidation, strains were grown to log phase in 100 ml of TGY and then incubated overnight with 100 mg of indole. Following incubation, cells were removed and the supernatants extracted twice with an equal volume of ethyl acetate. The ethyl acetate was evaporated *in vacuo* to a final volume of 5 ml, and 50 μ l spotted

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onto a silica thin layer chromatography (TLC) plate. Separation by TLC was carried out using ethyl acetate as the mobile phase. Commercial indigo, as well as indigo produced from *E. coli* (pDTG601a) incubations with indole, were used as controls.

Growth of D. radiodurans and Expression of TDO in the Presence of Radiation:

5 Strains MD560 and MD417 were grown in the presence of continuous γ -irradiation (60 Gy/hr) in a ^{137}Cs Gammacell 40 irradiation unit (Atomic Energy of Canada Ltd.) at room temperature (22°C). *E. coli* was used as a negative growth control for these experiments. Survival rates were determined by plating appropriate dilutions of irradiated cells and counting the colony forming units on plates. Strains MD560 and MD417 were grown in
10 the irradiation unit to an OD_{600} of 1.0 (1×10^8 cells/ml) and the cells were removed temporarily from the irradiator and concentrated to an OD_{600} of 5.0 (5×10^8 cells/ml). One ml of concentrated cells was aliquoted to each of five eleven milliliter vials and then 125 nmole of chlorobenzene added. Following addition of substrate, the vials were immediately placed back into the irradiator for incubation. One vial for each was removed
15 from the irradiator at 0, 20, 40, 60, and 120 minutes after addition of substrate. Promptly following removal from the irradiator, 0.5 ml of ethyl acetate was added, the sample vigorously shaken, and frozen at -70°C. While the aqueous portion was still frozen, the ethyl acetate fraction was removed, dried with anhydrous sodium sulfate and 1 μl was analyzed by GC.

20 *Resistance of D. radiodurans to Toluene and TCE:* *D. radiodurans* strains R1, MD560 and MD417 were grown overnight in liquid growth medium and then subcultured in duplicate to an OD_{600} of 0.02 in fresh medium with varying amounts of toluene or TCE added to each. After 18 hours incubation, the cell densities were determined and plotted as a function of solvent concentration.

25 *DNA manipulation:* DNA cloning, preparation and transformations were as described previously (Daly *et al.*, 1994a; 1995; 1996; Sambrook *et al.*, 1989).

Example 1

Production of a *D. radiodurans* strain which expresses toluene dioxygenase

Sequence Analysis of the D. radiodurans Genome: The nearly completed *D.*

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radiodurans genomic DNA sequence (<ftp://ftp.tigr.org/pub/data/d_radiodurans/>) was searched for similarity to TDO sequences using the Basic Local Alignment Search Tool (BLAST). No *D. radiodurans* sequences were found to have significant homology at the DNA level nor at the translated peptide level, suggesting that a TDO homolog does not exist in *D. radiodurans* strain R1 (wildtype).

Construction of D. radiodurans Strains MD560 and MD417: An *EcoRI*-*Bam*HI (4.2 kb) fragment containing the *todC1C2BA* genes (Zylstra *et al.*, 1989) was cloned from plasmid pHG2 (Wackett *et al.*, 1994) into the previously constructed *D. radiodurans* chromosomal tandem duplication vector pMD417 (Daly *et al.*, 1996) (Figure 1 and Experimental protocol) forming plasmid pMD532. pMD417 contains a single *EcoRI* and *Bam*HI site in the *tet* gene. By cloning *todC1C2BA* into the *EcoRI*-*Bam*HI sites of pMD417, the *tod* genes were placed under the control of a constitutive *D. radiodurans* promoter (Figure 1 and Experimental protocol). pMD532 was transformed into *D. radiodurans* R1 followed by selection on TGY plates (Daly *et al.*, 1994a) containing kanamycin. Strain MD560 was selected and the restriction map of its chromosomal integration site (Figure 1) was confirmed by Southern analysis (data not shown). The *tod* genes were present at about two copies per chromosome (8-20 copies per cell; *D. radiodurans* has 4-10 identical chromosomal copies per cell (Minton, 1994).

Expression of TDO in D. radiodurans: The *todC1C2BA* genes cloned into *D. radiodurans* (strain MD560) are constitutively expressed to make functional TDO. *D. radiodurans* strains R1 (wildtype), MD417 (*tod*⁻) and MD560 (*tod*⁺) were incubated with indole (Ensley *et al.*, 1983) and only strain MD560 yielded indigo. Incubation of 1.5×10^9 cells/ml of strain MD560 resulted in complete degradation of 25 nmole/ml of toluene and chlorobenzene in thirty minutes and at near equal rates (not shown). A similar reaction with 25 nmole/ml 3,4-dichloro-1-butene resulted in oxidation of about 40% of the substrate in eighty minutes. Incubations of overnight grown cultures of MD560 (1×10^8 cells/ml) with chlorobenzene, toluene and 3,4-dichloro-1-butene yielded the anticipated diol products, as determined by GC/MS analysis of culture supernatant extracts (see Experimental Protocol). All of the *in vivo* products were identical by GC/MS analysis to

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products obtained *in vitro* using purified toluene dioxygenase (data not shown). Although chlorobenzene, toluene, and 3,4-dichloro-1-butene were detectably oxidized by headspace analysis, 25 nmole/ml trichloroethylene (TCE), a substrate known to inactivate TDO (Lange *et al.*, 1997; Wackett *et al.*, 1989) was not detectably oxidized under these conditions. However, incubation of MD560 with volatile ^{14}C -TCE yielded a detectable increase in ^{14}C -nonvolatile material that was associated with the cells (not shown). This was consistent with previous studies *in vivo* (Wackett *et al.*, 1989) and *in vitro* (Li *et al.*, 1992) in which ^{14}C -TCE oxidation inactivates TDO and becomes covalently attached to cell materials. Strain MD417, lacking *tod* genes, was uniformly negative in the metabolism of all the TDO substrates tested.

Growth of D. radiodurans Strains and Expression of TDO in the Presence of Radiation: Over a period of thirty hours, *D. radiodurans*' growth characteristics and viability were not affected by the continual presence of 60 Gy/hr radiation in a ^{137}Cs irradiator (Figure 2). This level of continuous radiation exceeds those commonly found at waste sites (Riley *et al.*, 1992). *D. radiodurans* strains reached the stationary phase of their growth irrespective of the presence or absence of γ -irradiation. By comparison, *E. coli* did not grow and was killed by this level of radiation exposure, as expected. To test the ability of strain MD560 to functionally express TDO under irradiating conditions, strains MD560 and MD417 were grown in the irradiator (60 Gy/hour) for thirty hours to a cell density of 1.0×10^8 cells/ml. Following growth of both strains in the irradiator, each was concentrated on ice to 5×10^8 cells/ml and then incubated with 125 nmole/ml chlorobenzene in the presence and absence of radiation (60 Gy/hour, see Experimental Protocol). Strain MD560 oxidized 125 nmole/ml of chlorobenzene within one hour, irrespective of the presence or absence of radiation (Figure 3). Irradiator-grown control strain MD417, lacking the *tod* genes, was unable to degrade the chlorobenzene. The difference in rates observed for irradiated cells versus non-irradiated cell controls is an artifact and due to the way in which the experiment had to be conducted. The ^{137}Cs irradiator used in the experiment does not have a temperature control system and the irradiation experiments were, therefore, static and done at ambient room temperature (~22°C), whereas the non-irradiated controls were incubated in a 32°C incubator with

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shaking. This resulted in an apparent time-lag within the irradiator during which the cells warmed and the poorly-soluble substrate became uniformly mixed.

Resistance of D. radiodurans to toluene and TCE: The effects of solvent concentration on growth of *D. radiodurans* strains R1, MD417 and MD560 was tested.

- 5 The growth of *D. radiodurans* strains was not affected up to 800 mg/L for toluene and up to 1,500 mg/L TCE. These levels are well above those reported at sites (Riley *et al.*, 1992) containing contaminated groundwaters and many of those containing contaminated soil (Figure 4).

TDO was chosen for expression in *D. radiodurans* because it is prototypic of a
10 large class of bacterial dioxygenases and has a broad substrate range that includes compounds present at sites containing organic and radioactive mixed wastes. Furthermore, TDO is comprised of four protein components with their attendant metal and organic cofactors (Wackett, 1990) and, thus, its successful expression in *D. radiodurans* indicates that many less complex biodegradative enzyme systems can be expressed.

- 15 Strain MD560, expressing TDO, oxidized indole, toluene, chlorobenzene, and 3,4-dichloro-1-butene, all known substrates for TDO. *D. radiodurans* strains grew under continuous irradiating conditions of 60 Gy/hr in a ¹³⁷Cs irradiator (Figure 2). Furthermore, strain MD560 synthesized functional TDO under those conditions (Figure 3) and degraded 125 nmole/ml chlorobenzene while being exposed to radiation.

- 20 The cell envelope of *D. radiodurans* includes an outer and inner lipid membrane that surrounds the cell wall (Thompson *et al.*, 1982a; 1982b). The results presented herein indicate that the membrane architecture of this organism does not result in extreme sensitivity to organic solvents. Organic solvents are generally toxic to bacteria by making their membranes porous (deSmet *et al.*, 1978; Sikkema *et al.*, 1995). Toluene and TCE are
25 two of the most common organopollutants at radioactive DOE waste sites (Riley *et al.*, 1992); toluene has been reported as high as 26 mg/L groundwater and 2,000 mg/kg soil, and TCE as high as 1,000 mg/L and 12,000 mg/kg. *D. radiodurans* strains R1, MD560 and MD417 were all found to be naturally tolerant to toluene and TCE groundwater concentrations well above those found at most sites, and resistant to about half the highest
30 toluene concentrations reported in contaminated soils (Figure 4).

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The lack of TCE oxidation beyond that measured using sensitive ^{14}C assay has been attributed to turnover-dependent TDO inactivation (Wackett *et al.*, 1989). Enzymatic TCE oxidation is known to generate reactive acyl chlorides that bind covalently to proteins and other macromolecules (Li *et al.*, 1992). Sustained biological TCE oxidation may, thus, can be accomplished by the intracellular biosynthesis of a scavenging nucleophile, such as glutathione, to protect against enzyme inactivation. Our analysis of genome sequences failed to detect DNA homologues to *E. coli* genes *gshA* and *gshB* (not shown). Accordingly, these genes can be cloned and expressed in *D. radiodurans* or other strains of the invention.

10

Example 2

Production of a *D. radiodurans* strain which expresses the *mer* operon

Construction and characterization of mercury (II) resistant D. radiodurans strains:

The cloned *mer* operon encodes six proteins that confer mercury resistance functions on *E. coli* (Hamlett *et al.*, 1992) (Figure 7, top). The entire *D. radiodurans* genomic DNA sequence (White, 1999) was searched for similarity to these *mer* operon sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997). No authentic *mer* operon was identified in the *D. radiodurans* genome. Moreover, no orthologs of the *mer* genes were found, except *merR*- and *merD*-like genes, that are transcriptional regulators encoded in all known bacterial genomes (Schiering *et al.*, 1991). This *D. radiodurans* genomic analysis is supported by our experimental results showing the sensitivity of wildtype *D. radiodurans* strain R1 to mercury.

Strain MD735: The *mer* operon was cloned into the *D. radiodurans* autonomously replicating plasmid pMD66 (Daly *et al.*, 1994a) forming MD727, and transformed into strain R1 (MD735, Figure 7A). Briefly, *D. radiodurans* and *E. coli* strains were grown in TGY medium and Luria-Bertani (LB) medium, respectively, with aeration on rotary shakers at 32°C and 37°C, respectively. Kanamycin (Km) and chloramphenicol (Cm) were used at a concentration of 8 µg/ml and 3 µg/ml, respectively, for recombinant *D. radiodurans* strains. Freshly prepared Merbromin (mercurochrome; 2',7'-Dibromo-5'[hydroxymercuri]-fluorescein) was used in the following concentrations, for growth on

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solid medium, 30 µg/ml; in liquid medium, 50 µg/ml. Merbromin was used in our Hg (II) studies, over HgCl₂ because its red color (Figure 2B) allowed us to track highly toxic Hg (II) waste more easily. This construction placed the *mer* genes under the control of a constitutive *D. radiodurans* promoter (P2, Figure 7A), and Southern analysis showed that the *mer* operon was present at about one copy per cell (Figures 9A, 9B).

Strain MD736: This strain has the *mer* operon integrated into the previously described chromosomal S11 locus (Smith *et al.*, 1988), located on the 2.8 Megabase pair (Mbp) chromosome (Chromosome I [White, 1999]) of *D. radiodurans* (position 1,677,743 - 1,689,109), as a tandem duplication. The functional difference between the *mer*-containing tandem duplication vector and the *mer*-containing autonomous replicating plasmid is that the plasmid origin of replication segment (dORI; Figure 7A) was replaced with a 4 kb internal segment of the *D. radiodurans* chromosomal S11 locus, bc (Figure 7B). This 4 kb chromosomal segment allows it to recombine into the targeted S11 chromosome sequence (bc, Figure 7B) by a single crossover; without integration, a vector lacking a dORI sequence cannot replicate in the cell and is lost. Upon integration, the *mer* operon within this tandem duplication became flanked by 4 kb bc repeats. In strain MD736, the *mer* operon was present at about ten copies per cell (Figure 9A, 9B). It should be noted that the presence of the 18 kb *EcoRI* fragment (Figure 7B) visualized by Southern Blotting (Figure 9B) supports the presence of at least two copies on a chromosome. The fact that we detected only about ten copies per cell indicates that the strain is not homozygous; under the described culture conditions (Figure 7B), some of the 8-10 haploid copies per cell (Hansen, 1978) of Chromosome I apparently lack this insertion.

Strain MD737: Whereas the tandem duplication strain (MD736) has about ten *mer* copies per cell, amplification vectors like pS11 (Smith *et al.* 1988), integrate themselves at 150-200 vector copies per cell. When integrated, the duplicated chromosomal flanking sequences of pS11 (11.4 kb) (abcd, Figure 7C), can readily recombine with identical insertions on other chromosomes, leading to amplification - presumably by uneven homologous recombination of daughter chromosomes (Smith *et al.* 1988). The salient functional difference between a duplication vector (*e.g.*, in MD736) and an amplification vector (*e.g.*, in MD737) is that an amplification vector lacks a

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deinococcal constitutive promoter (P1, Figure 7B) upstream of the antibiotic resistance marker (Km^R, Figure 6C). Without such a promoter, the only cells able to grow under selective antibiotic conditions are those that have highly amplified antibiotic resistance determinants (Smith *et al.* 1988). The situation in MD737 is analogous because specific transcription signals upstream of both the Km^R resistance gene (*aphA*) and the *mer* operon are lacking (Figure 7C), leading to amplification with selection pressure (Figures 7C, 9A, 9B). In strain MD737, the multiple copies of Chromosome I each contain an average of about 15 copies of the *mer* operon (~150 copies per cell) (Figures 9A, 9B).

Strain MD767: Unlike the tandem duplication and amplification vectors, a vector integrated into a host cell's chromosome by direct-insertion becomes a permanent and unchanging fixture in the cell's genome. The permanence of this integration arrangement is a result of the unique chromosomal DNA sequences flanking the integrated vector, that will not recombine (ABC and DEF, Figure 8A). This system of integration is not versatile like a tandem duplication or amplification integration since the number of integrated copies cannot be altered independently of the chromosome number. This inflexibility is a potential disadvantage for recombinant cells containing a direct insertion since they are less able to adapt to changing environmental conditions. However, the potential advantage in using this cloning approach is the fact that the insertion cannot be lost, even in the absence of any selection. And, from an environmental release standpoint, direct insertions are less likely to be transferred to other indigenous microorganisms.

Direct insertions of a plasmid in *D. radiodurans* Chromosome I have previously been constructed (Daly *et al.*, 1995). The construction of plasmids suitable for this sort of integration, however, is very labor-intensive (Daly *et al.*, 1995). A simple alternative approach to constructing a *D. radiodurans* strain containing a direct insertion of the *mer* operon was to use a genetic technique that takes advantage of this organism's transformation and recombination capabilities, and the common structural backbone of our transforming vectors (see Figure 8). This is the first demonstration in *D. radiodurans* showing how a gene cloned as a duplication insertion can be converted to a direct (permanent) insertion by recombinative transformation. Following selection for mercury resistance (Figure 8B), individual clones were pre-screened for resistance to

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chloramphenicol to select for crossovers in the desired chromosomal regions, yielding strain MD767 (Figure 7C), that contains one *mer* operon per 2.8 Mbp-chromosome (Figures 8A, 8B).

mer copy number: The copy number of the *mer* operon in each of the engineered strains (MD735, MD736, MD737, and MD767) was determined (Figures 9A, 9B; the agarose gel is aligned with the Southern blot). The *merA* copy number in these four strains, growing exponentially, was determined by comparing the number of radioactive disintegrations of each hybridizing band to the *merA*-specific band of strain MD767 (Figure 9B), that contains about ten copies of the *mer* operon per cell; the direct insertion is located on Chromosome I, that exists at 8-10 copies per exponentially growing cell (Hansen, 1978). The approximate number of *merA* copies per cell in: R1 = 0; MD735 = 1; MD767 = 10; MD736 = 10; MD737 = 150. An exponentially growing *D. radiodurans* cell contains about five times the DNA content of an *E. coli* stationary-phase cell (Krasin et al., 1977). Taking this into consideration, it is estimated from data shown in Figure 9A, B that *E. coli* BL308 has about 20-30 *mer* copies per cell.

Resistance to mercury (II): The engineered *D. radiodurans* strains, as well as the *E. coli* strain BL308 (24), were inoculated into liquid medium containing 50 μ M Merbromin or 50 μ M HgCl₂ and growth for each was monitored over a period of thirteen days (Figure 9D). Wildtype *D. radiodurans* did not grow in the presence of 50 μ M Hg (II) and was inhibited by 10-15 μ M Merbromin or HgCl₂. The strains containing the cloned *mer* operon were variably affected by Hg (II) showing the following order of resistance: BL308 > MD737 > MD736 > MD735 > MD767 > R1 (Figure 9D). It should be noted that the normal growth rate of *E. coli* is about four times that of *D. radiodurans*. The five-day lag phase observed before the onset of growth of *mer*-containing *D. radiodurans* cells in 50 μ M Hg (II) (Figure 9D) could be shortened to one day by inoculating Hg (II)-containing medium with cells pre-grown in the presence of 5 μ M Hg (II). The growth rates and final cell densities of these pre-induced *D. radiodurans* cultures did not differ significantly from those shown in Figure 9D. This result supports that the *mer* genes are being induced in *D. radiodurans*. The most mercury resistant *D. radiodurans* strain MD737 was examined to determine the highest Hg (II) concentration at which growth

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could be sustained. It was found that MD737 cells pre-cultured in Hg (II) could subsequently grow in medium containing up to 100 μ M Hg (II) (data not shown).

Strain MD736 is more resistant to Hg (II) than MD767, even though they apparently have the same *mer* copy number (Figures 9A, 9B). The copy number of the tandem duplication- and direct- insertions in *D. radiodurans* are very similar (Daly *et al.*, 1995). As described above, direct insertions are much less versatile than tandem duplications and the strain MD736 is likely to be heterozygous; where some of the Chromosome I copies apparently lack the *mer* tandem duplication. The ability of strain MD736 to further increase its number of *mer* tandem duplications with mercury (II) selection was tested. By increasing the mercury concentration over a range of 0-35 μ M, the copy number of the *mer* operon in MD736 doubled, compared to growth with just Km selection (Figure 9C). MD767, containing the direct *mer* insertion did not show a change in copy number with increasing mercury selection, compared to selection with Km (Figure 9C).

Effect of γ -radiation: To determine any effect of continuous exposure to γ -radiation on the growth of these strains in the presence of Hg (II), we tested the strains R1 (*recA*⁺, *mer*⁻), *rec30* (*recA*⁻, *mer*⁻; Daly1), MD735 (*recA*⁺, *mer*⁺), MD736 (*recA*⁺, *mer*⁺), MD737 (*recA*⁺, *mer*⁺), MD767 (*recA*⁺, *mer*⁺), and the wildtype *E. coli* strain K12 (*recA*⁺) containing pDB7 (BL308 [24]) (*mer*⁺) (Figure 10). After four days of incubation in an irradiating (60 Gy/hr) and mercury (II)-containing environment, as expected, the only strains that could grow in the presence of both were MD735, MD736, MD737, and MD767 (Figure 10D).

Example 3

Construction of a toluene-metabolizing and Hg (II) resistant

D. radiodurans strain MD764.

To assess the potential for expressing multiple remediating functions, encoded on separate gene cassettes, in *Deinococcus*, a strain expressing both mercury resistance/reducing and toluene metabolizing functions (Figure 9D, 11) was constructed. The genetic procedure of integrating two different gene cassettes at the same *D.*

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radiodurans genomic locus has been described previously (Daly *et al.*, 1996), but in the context of analyzing recombination following high dose irradiation, and not gene expression.

Strain MD764 was analyzed for its ability to resist (Figure 9D) and reduce Hg (II) (Figures 12, 13) as well as metabolize the TDO specific substrate toluene (Figure 11D). The growth characteristics of strain MD764 in Hg (II), in the presence and absence of radiation (60 Gy/hr) were indistinguishable from those expressed in the parent strain MD737 (Figure 11C). MD764 could also reduce Hg (II) to Hg (0) (Figures 12, 13), in a manner similar to, if not indistinguishable from, MD737.

TDO activity was measured by ultraviolet (UV) absorbance and thin layer chromatography: Mercury (II), and the expression of *mer* operon genes, did not erode the ability of recombinant *D. radiodurans* cells to express functional toluene dioxygenase activity. This was demonstrated with strain MD764 when toluene was provided as the substrate (Figure 11D). Toluene dioxygenase oxidizes toluene to *cis*-1,2-dihydroxy-3-methylcyclohexa-3,5-diene (*cis*-toluene dihydrodiol), which absorbs maximally at 264 nm, and substantial absorbance at this wavelength was observed in culture supernatants of *D. radiodurans* MD764 (Figure 11D) containing the recombinant mercuric reductase and toluene dioxygenase genes (Figure 11B), but not in the wild-type strain *D. radiodurans* R1, lacking both of those gene cassettes (Figure 11D). From the extinction coefficient (33), the apparent dihydrodiol product was present at a concentration of about 1 mM at 31 hours. The putative product was greater at 31 hours than at 106 hours. These data were supported by direct observation of a product by TLC in comparison with authentic *cis*-toluene dihydrodiol (Figure 11D, inset). After 20 hours, a single metabolite (Figure 11D, inset, lane 2) comigrating with the dihydrodiol standard (Figure 11D, inset, lane 1) was observed. The amount of the dihydrodiol product decreased upon further incubation, consistent with the product data obtained by UV spectroscopy. The *cis*-dihydrodiol product of toluene dioxygenase is dehydrogenated by *D. radiodurans* to a product with a mass spectrum identical to 3-methylcatechol (unpublished). The data in Figure 10D (top) is consistent with this as the UV absorption maximum of the product(s) in the growth medium of strain MD764 shifts from that of a dihydrodiol (λ_{max} at 264 nm) at 31 hours to

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that of a catechol (λ_{max} at 270 nm) at 106 hours.

Reduction of Hg (II) to volatile elemental Hg (0) by engineered strains.

Mercuric reductase assays: MerA activity was determined in cell extracts of *D. radiodurans* strains R1, MD767, MD735, MD736, MD737 and MD764 by following Hg (II)-stimulated NADPH oxidation spectrophotometrically (Schottel, 1978). Mercury (II)-dependent NADPH oxidation was observed in cell extracts of recombinant strains containing the *merA* gene, but not in wildtype *D. radiodurans* strain R1 (Figure 12A). In the absence of Hg (II), the rate of NADPH oxidation by the *mer* containing strains was comparable to that of strain R1 (Figure 12A, curve A). Also, there was good correlation between the variable Hg (II)-dependent NADPH oxidation activity (Figure 12A) and the resistance of strains to Hg (II) (Figure 9D).

MerA was visualized by SDS-polyacrylamide gel electrophoresis (PAGE) (Figure 12B). Cell extracts from strains R1, MD767, MD735, MD736, MD737, and MD764 were subjected to Orange A dye affinity chromatography as described previously (Schottel, 1978). After extensive column washing, the mercury-dependent NADPH oxidation activity was eluted from the column using NADPH; a process that increased the specific activity about 20-fold, and is an amount comparable to results from the same procedure used to purify MerA from other bacteria. SDS-PAGE analysis of the fractions containing MerA showed enrichment of two major proteins with approximate molecular weights of 62 kDa and 54 kDa in *merA*-containing strains, but not in the control strain R1 (wildtype). Two bands of similar molecular weights associated with mercuric reductase activity have been described for both the *E. coli* reductase (Nakamura *et al.*, 1988) and the *P. aeruginosa* enzyme (Schottel, 1978); the ratio of the two bands shifted depending on the storage of the protein, but with no significant change in specific activity. It is likely that the 54 kDa band is a proteolytic fragment of the larger.

Mercury volatilization: The observed mercuric ion dependent NADPH oxidation suggests the concomitant production of volatile Hg (0) by the *mer*-containing *D. radiodurans* strains. An X-ray film assay (38) (Figure 12C) was used for detecting the

-44-

production of Hg (0) vapor by the incubation of the *D. radiodurans* strains with Hg (II). There was a good correlation of results between the Hg (II) resistance profiles of these engineered strains containing the *mer* operon (Figure 9D) and the X-ray film results (Figure 12C); elemental mercury vapor reacts with the silver ions of X-ray film, causing film darkening. Following 14 hours of strain incubation with Hg (II) in the microplate, covered with an X-ray film, only those strains containing the *mer* operon caused film exposure (Figure 12C). Incubation of cells with either Merbromin or HgCl₂ gave similar results, except that the film exposure was reduced when using Merbromin. Incubation of Hg (II) with the controls, R1 (wildtype), MD744 (*mer*⁻, *tod*⁺, Cm^r), MD560 (*mer*⁻, *tod*⁺, Km^r), or growth medium alone, did not show any evidence for Hg (0) volatilization.

Mercury depletion was determined in open cultures of *D. radiodurans* MD764 and the control strain R1 by cold vapor atomic fluorescence spectroscopy (CVAFS) (Figure 13). This experiment used CVAFS to detect total mercury in the system by analyzing the concentration of Hg (0) after quantitative reduction of Hg (II) to Hg (0), using stannous chloride. The bacterial cultures were open to the atmosphere for two hours before analysis, during which time biologically reduced Hg (II) would be anticipated to be lost from the system. The levels of mercury used were necessarily low because of the sensitivity of the CVAFS method. As shown in Figure 13, *D. radiodurans* strain MD764, representative of the *merA*-containing strains, was observed to expel mercury from the culture which it did to near baseline level in two hours. The control strain culture, lacking *merA*, showed no measurable loss of mercury over the same two hours. Although the cells were pre-incubated in medium without mercury for two hours and washed several times before the experiment was initiated, the level of mercury retained in the MD764 cells grown in the presence of HgCl₂ was more than twice the mercury added during the experiment. This sequestration of mercury did not significantly deplete over the course of the experiment. By increasing the length of the pre-incubation period to several hours and washing the cells exhaustively, the background level was reduced by up to 75%. However, the rate of mercury depletion was diminished to the level observed for cells not previously grown in the presence of Hg (II) (data not shown). Mercuric reductase specific activity in cell extracts from cells grown without Hg (II) is less than one half that of cell

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extracts when the cells were grown in the presence of Hg (II) has also been observed.

Example 4

Development of *D. radiodurans* for Growth on/ Metabolism in DOE Mixed Wastes

Recombinant strains of *Deinococcus* engineered for bioremediation of mixed
5 wastes may be prepared as a library of individual (primary) *Deinococcus* strains, each
containing a different toxin-degrading gene cassette or metal resistance gene/s which has
been cloned into Type-II, Type-III or Type-IV insertional vectors and separately
transformed into *Deinococcus*. These primary stains are used in bioremediation
compositions individually or combined depending on the composition of a particular waste
10 site.

The primary isolates also serve as *Deinococcus* chromosomal reservoirs for toxin-
remediating genes and metal resistance genes and provide a source of DNA that is
subsequently transformed and combined into different strains to meet the requirements of
surviving in and remediating a large variety of sites. For instance, genes necessary for
15 metal resistance are cloned into *D. radiodurans* using Type-II (direct-insertion) vectors as
described above. These metal resistance genes then become a permanent fixture in the
cells' chromosomes and are not lost by recombinational 'pop-out' because the genes are be
flanked by non-repetitive DNA. Because of the large variety of organic toxin-degrading
genes available and the potential advantages of forming hybrid clusters that could be
20 amplified for high level expression, integration of toxin-degrading genes into metal
resistant strains is done using Type-III or Type-IV (duplication-insertion) vectors.
Maintenance of the primary library strains is done with single antibiotic resistance markers
and transformation derivatives containing different gene cassettes are maintained by
multiple drug selection. In the case of cloning organic toxin-catabolizing genes into *D.*
25 *radiodurans*, an alternative to selection with antibiotics is selection for a strain's ability to
grow on or catabolize a specific organic substrate.

The ability of *D. radiodurans* to grow or catabolize one or more organic substrates,
is selected for using minimal media (MM) supplemented with 0.0002% L-methionine in
combination with required vitamins, minerals and nucleic acids as is known in the art. For

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instance, MM is prepared as described above with the addition of toluene, biphenyl or metabolic intermediates of toxin degradation. This powerful selection, called 'shuffle-selection' allows *D. radiodurans* itself to 'choose' (by transformation and selection on MM plus a toxin) its own combination of genes that may allow growth. This gives the *D.*

5 *radiodurans* strains the opportunity to construct its own metabolic pathways from the many catabolic genes presented with at the time of transformation. Further, by virtue of the repetitive chromosomal sequences flanking the duplication-insertion vectors, *D.*

radiodurans amplifies those genes required for higher expression levels. The isolated strains that can grow on MM plus a toxin, may then be analyzed to determine what genes

10 were selected and to what extent they were amplified for expression.

Example 5

D. geothermalis was transfected with plasmid pMD66 originally designed fro *D. radiodurans* (Daly, 1994a). pMD66 contains a *D. radiodurans* origin of replication and two distinct *Deinococcus* promoters from expressing cloned genes. These elements are

15 functional in *D. geothermalis* growing at 50°C (Figure 14). DNA prepared from the indicated strains was digested with *EcoRI*, electrophoresed and subjected to Southern blotting with a radiolabeled pMD66 probe. Lanes: 1, λ phage cut with *HindIII*; 2, *D. geothermalis* wild type; 3, *D. radiodurans* R1; 4, *D. geothermalis* + pMD66; 5, *D. radiodurans* + pMD66; 6, purified pMD66 cut with *EcoRI*.

20 Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.

References

- "Linking Legacies Report" U.S. Department of Energy. Office of Energy Research, Subsurface Science Program, Washington D.C., DOE/EM-319, (1992).
- 5 "Nuclear waste problems associated with DOE's inactive waste sites" U.S. General Accounting Office (GAO), U.S. Government Accounting Office, Washington D.C., GAO/RCED-88-229FS (1988).
- Focht DD. "Aerobic biotransformation of polychlorinated biphenyls" in Hurst *et al. Manual of Environmental Microbiology* (ASM Press) (1995).
- 10 Leisinger T, Cook AM, Hutter R, Nuesch J. (eds.). "Microbial degradation of xenobiotic and recalcitrant compounds" (Academic Press, New York, NY) (1981).
- J, Hazen T, Benson S, Blaine-Metting F, Palmisano AC. "Bioremediation of metals and radionuclides" (Office of Biological and Environmental Research, U.S. Department of Energy, Germantown, MD) (1999).
- 15 Murray RGE. "The family *Deinococcaceae*" in *The prokaryotes*. Balows A, Truper HG, Dworkin M, Harder W, Schleifer, KH. (eds.) vol 4, 2nd ed. (Springer-Verlag, New York, NY) (1992).
- Riley RG, Zachara JM, Wobber FJ. DOE/ER "Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface science research" (U.S. Department of Energy. Office of Energy Research, Subsurface Science Program, 20 Washington D.C.) (1992).
- Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: A laboratory manual*, 2nd ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) (1992).
- U.S. General Accounting Office (GAO). 1988. Nuclear waste problems associated with DOE's inactive waste sites. GAO/RCED-88-229FS, U.S. Government Accounting 25 Office, Washington D.C.
- Wackett LP. "Biodehalogenation: Natural and engineered systems" in Hinchee R. ed. *Proceedings of the Third International In Situ and On-Site Bioreclamation Symposium* (1997).
- Altschul *et al.* Nucleic Acids Res. 25, 3389 (1997).
- 30 Barrineau *et al.* J. Molec. Appl. Genet. 2, 601 (1984).
- Bloom NS, Crecelius EA. Marine Chemistry 14, 49 (1983).
- Brooks *et al.*, Int. J. Syst. Bacteriol. 30, 627 (1980).

- Carrol JD, Daly MJ, Minton KW. *J. Bacteriol.* 178, 130 (1996).
- Chakrabarty AM. *ASM News*, 62, 130 (1996).
- Creighton E., "The Proteins", (WH Freeman, New York, 2nd ed.) (1993).
- Daly MJ, Minton KW. *J. Bacteriol.* 177, 5495 (1995).
- 5 Daly MJ, Minton KW. *J. Bacteriol.* 178, 4461 (1996).
- Daly MJ, Minton KW. *Gene*, 187, 225 (1997).
- Daly MJ, Ouyang L, Fuchs P, Minton KW. *J. Bacteriol.* 176, 3508 (1994a).
- Daly MJ, Ouyang L, Minton KW. *J. Bacteriol.* 176, 7506 (1994b).
- de Smet MJ, Kingma J, Witholt B. *Biochem. Biophys. Acta*, 506, 64 (1978).
- 10 Diels *et al.* *J. Ind. Microbiol.* 14, 142 (1995).
- Dowling DNR, Pipke R, Dwyer DF. *FEMS Microbiol. Let.* 113, 149 (1993).
- Ensley *et al.* *Science* 222,167 (1983).
- Fox B, Walsh CT. *J. Biol. Chem.* 257, 2498 (1982).
- Gibson DT, Cardini GE, Maseles FC, Kallio RE. *Biochemistry*, 9, 1631 (1970).
- 15 Gorby YA, Lovley DR. *Environ. Sci. Technol.* 26, 205 (1992).
- Hamlett *et al.* *J. Bacteriol.* 174, 6377 (1992).
- Hansen MT. *J. Bacteriol.* 134, 71 (1978).
- Higham DP, Sadler PJ, Scawen MD. *Science*, 225, 1043 (1984).
- Infante, P. F., and T. A. Tsongas. *Environ. Sci. Res.* 25, 301 (1982).
- 20 Jacobson JL, Jacobson SW. *N. Engl. J. Med.* 335, 783 (1996).
- Ji G, Silver S. *J. Bacteriol.* 174, 3684 (1992).
- Kobal VM, Gibson DT, Davis RE, Garza A. *J. Am. Chem. Soc.* 95, 4420 (1973).
- Krasin F, Hutchinson F. *J. Mol. Biol.* 116, 81 (1977).
- Lange CC, Wackett LP, Minton KW, Daly MJ. *Nature Biotech.* 16, 929 (1998).
- 25 Lange CC, Wackett LP. *J. Bacteriol.* 179, 3858 (1997).
- Li S, Wackett LP. *Biochem. Biophys. Res. Comm.* 58, 2820 (1992).
- Lovely DR. *J. Ind. Microbiol.* 14, 85 (1995).
- Lovley DR. *Microbiol. Rev.* 55, 259 (1991).
- Macilwain C. *Nature*, 383, 375 (1996).
- 30 Mattimore V, Udupa KS, Berne GA, Battista JR. *J. Bacteriol.* 177, 5232 (1996).

-49-

- McKay DM, Cherry JA. *Environ. Sci. Technol.* 23, 630 (1989).
- McKlveen JW. *Australas Phys. Eng. Sci. Med.*, 13, 101 (1990).
- Minton KW. *Molec. Microbiol.* 13, 9 (1994).
- Minton KW. *Mutat. Res. DNA Repair* 362, 1 (1996).
- 5 Misra TK. *Plasmid* 27, 4 (1992).
- Mondello FJ. *J. Bacteriol.* 171, 1725 (1989).
- Moseley BEB, Evans DM. *J. Gen. Microbiol.* 129, 2437 (1983).
- Nakamura K, Nakahara H. *Appl. Environ. Microbiol.* 54, 2871 (1988).
- Nies DH, Silver S. *J. Ind. Microbiol.* 14, 186 (1995).
- 10 Rainey *et al.* *Int. J. Syst. Bacteriol.* 47, 510 (1997).
- Rugh CL, Senecoff JF, Meagher RB, Merkle SA. *Nature Biotech.* 16, 925 (1998).
- Rugh *et al.* *Proc. Natl. Acad. Sci. USA*, 93, 3182 (1996).
- Schiering *et al.* *Nature*, 352, 168 (1991).
- Schottel JL. *J. Biol. Chem.* 253, 4341 (1978).
- 15 Sikkema J, de Bont JA, Poolman B. *Microbiol. Rev.* 59, 201 (1995).
- Smith MD, Lennon E, McNeil LB, Minton KW. *J. Bacteriol.* 170, 2126 (1988).
- Thompson BG, Murray RGE. *Can. J. Microbiol.* 28, 1081 (1982).
- Thompson BG, Murray RGE. *Can. J. Microbiol.* 28, 522 (1982).
- Thornley MJ. *J. Appl. Bacteriol.* 26, 334 (1963).
- 20 Tsapin *et al.* *J. Bacteriol.* 178, 6386 (1996).
- Turner JS, Robinson NJ. *J. Ind. Microbiol.* 14, 119 (1995).
- Venkateswaran, A. *et al.*, *Applied Environ. Microbiol.* 66(6):2620-2626 (2000).
- Voordouw G, Brenner S. *Eur. J. Biochem.* 159, 347 (1986).
- Wackett LP, Gibson DT. *Appl. Environ. Microbiol.* 54, 1703 (1988).
- 25 Wackett LP, Householder SR. *Appl. Environ. Microbiol.* 55, 2723 (1989).
- Wackett LP, Sadowsky MJ, Newman LM, Hur HG, Li S. *Nature* 368, 627 (1994).
- Wackett LP. *Methods Enzymol.* 188, 39 (1990).
- White O. manuscript under review, *Science* (1999).
- Ziffer *et al.* *Tetrahedron* 33, 2491 (1977).
- 30 Zylstra GJ, Gibson DT. *J. Biol. Chem.* 264, 14940 (1989).

WHAT IS CLAIMED:

1. A radiation resistant bacterium engineered to detoxify at least one toxin.
2. A radiation resistant bacterium of claim 1 which survives acute exposure to ionizing radiation of up to about 15,000 Gy or can grow in the presence of continuous radiation of about 60 Gy/hour.
3. A radiation resistant bacterium of claim 1, wherein the bacterium is selected from the group consisting of *Enterococcus*, *Alcaligenes* and *Deinococcus*.
4. A radiation resistant bacterium of claim 1, wherein the toxin is selected from the group consisting of radionuclides, heavy metals and organic compounds.
5. A radiation resistant bacterium of claim 4, wherein the radionuclide is $^{235}\text{Uranium}$ (γ , α)^E, $^{90}\text{Strontium}$ (β -)^E, $^{238}\text{Plutonium}$ (α)^E, $^{137}\text{Cesium}$ (γ , β -)^E and $^{99}\text{Technetium}$ (β -)^E.
6. A radiation resistant bacterium of claim 4, wherein the heavy metal is chromium, lead, arsenic, zinc, cadmium, cobalt or mercury.
7. A radiation resistant bacterium of claim 4, wherein the organic compound is a chlorinated hydrocarbon, fuel hydrocarbon or polychlorinated biphenyl.
8. A radiation resistant bacterium of claim 7, wherein the chlorinated hydrocarbon is trichloroethylene.
9. A radiation resistant bacterium of claim 7, wherein the fuel hydrocarbon is toluene.

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10. A radiation resistant bacterium of claim 7, wherein the polychlorinated biphenyl is Arochlor.

11. A radiation resistant bacterium of claim 1, wherein the toxin is selected from the group consisting of pesticides, toxicants, agricultural chemicals, algicides, fungicides, sterilants, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, greasy wastes, solvents, crude oil, diesel fuel, waste oil, Bunker "C" oil, phenolics, halogenated hydrocarbons, terpene alcohols, anthracene, chlorotoluenes, chrysene, cresols, di-N-octylphthalate, dichlorobenzene, dichlorethanes, dichloropropanes, dichlorotoluene, 2-ethoxyethanol, ethylene glycol, ethylene glycol monoethyl ether acetate, ethylbenzene, fluorene, isoprenoids, methyl ethyl ketone, methylene chloride, naphthalene, pentachlorophenol, phenanthrene, 1,1,2,2-tetrachloroethane, toluene, 1,1,2-trichloroethane, trichloroethylene, benzoate, chlorobenzoates, methanol, ethyl acetate, cyclohexanone, ethylbenzene, 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, m,o,p-xylene, butyl acetate, camphor, hexane, heptane, octane, nonane, d-limonene, linalool, geraniol, citronellol.

12. A radiation resistant bacterium of claim 1, wherein the bacterium has been engineered to express a heterologous protein or enzyme selected from the group consisting of toluene dioxygenase, the proteins encoded by the *mer* operon, the proteins encoded by the *Pseudomonas* Tol region, the proteins encoded by the *xylL-xyIE* operon, a
5 monooxygenase, the proteins encoded by *bphA1A2A3A4*, the proteins encoded by *czcA*, B and C genes, the *smtA* and B genes and the *arsA* and B genes.

13. A radiation resistant bacterium engineered to detoxify at least two toxins.

14. A radiation resistant bacterium of claim 13 which survives acute exposure to ionizing radiation of up to about 15,000 Gy or can grow in the presence of continuous radiation of about 60 Gy/hour.

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15. A radiation resistant bacterium of claim 13, wherein the bacterium is *Deinococcus radiodurans*.
16. A radiation resistant bacterium of claim 13, wherein the toxin is selected from the group consisting of radionuclides, heavy metals and organic compounds.
17. A radiation resistant bacterium of claim 16, wherein the radionuclide is $^{235}\text{Uranium } (\gamma, \alpha)^{\text{E}}$, $^{90}\text{Strontium } (\beta)^{\text{E}}$, $^{238}\text{Plutonium } (\alpha)^{\text{E}}$, $^{137}\text{Cesium } (\gamma, \beta)^{\text{E}}$ and $^{99}\text{Technetium } (\beta)^{\text{E}}$.
18. A radiation resistant bacterium of claim 16, wherein the heavy metal is chromium, lead, arsenic, zinc, cadmium, cobalt or mercury.
19. A radiation resistant bacterium of claim 16, wherein the organic compound is a chlorinated hydrocarbon, fuel hydrocarbon or polychlorinated biphenyl.
20. A radiation resistant bacterium of claim 19, wherein the chlorinated hydrocarbon is trichloroethylene.
21. A radiation resistant bacterium of claim 19, wherein the fuel hydrocarbon is toluene.
22. A radiation resistant bacterium of claim 19, wherein the polychlorinated biphenyl is Arochlor.

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23. A radiation resistant bacterium of claim 13, wherein the toxin is selected from the group consisting of pesticides, toxicants, agricultural chemicals, algicides, fungicides, sterilants, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, greasy wastes, solvents, crude oil, diesel fuel, waste oil, Bunker "C" oil, phenolics, halogenated
5 hydrocarbons, terpene alcohols, anthracene, chlorotoluenes, chrysene, cresols, di-N-octylphthalate, dichlorobenzene, dichlorethanes, dichloropropanes, dichlorotoluene, 2-ethoxyethanol, ethylene glycol, ethylene glycol monoethyl ether acetate, ethylbenzene, fluorene, isoprenoids, methyl ethyl ketone, methylene chloride, naphthalene, pentachlorophenol, phenanthrene, 1,1,2,2-tetrachloroethane, toluene, 1,1,2-trichloroethane,
10 trichloroethylene, benzoate, chlorobenzoates, methanol, ethyl acetate, cyclohexanone, ethylbenzene, 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, m,o,p-xylene, butyl acetate, camphor, hexane, heptane, octane, nonane, d-limonene, linalool, geraniol, citronellol.

24. A radiation resistant bacterium of claim 13, where the bacterium has been engineered to express a heterologous protein or enzyme selected from the group consisting of toluene dioxygenase, the proteins encoded by the *mer* operon, the proteins encoded by the *Pseudomonas* Tol region, the proteins encoded by the *xyiL-xyiE* operon, a
5 monooxygenase, the proteins encoded by *bphA1A2A3A4*, the proteins encoded by *czcA*, B and C genes, the *smtA* and B genes and the *arsA* and B genes.

25. A bioremediation composition comprising a bacterium of any one of claims 1-24.

26. A bioremediation composition of claim 25 further containing an agent selected from the group consisting of a film forming agent and a nutrient agent.

27. A bioremediation composition of claim 25 which if formulated for controlled release.

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28. A bioremediation composition of claim 26 which if formulated for controlled release.
29. A *Deinococcus radiodurans* strain which metabolizes toluene and is resistant to mercury.
30. The *Deinococcus radiodurans* strain of claim 29 which expresses the *P. putida todC1C2BA* and *E. coli merA* operons.
31. A method of bioremediation, comprising the step of exposing a sample to a composition of 25.
32. A method of bioremediation, comprising the step of exposing a sample to a composition of any one of claims 26-28.
33. A method of claim 32, wherein the sample is contaminated with radionuclides.
34. A method of claim 33, wherein the composition is released into a waste site.
35. A bioremediation composition comprising a bacterium of claim 30.
36. A bioremediation composition of claim 35 further containing an agent selected from the group consisting of a film forming agent and a nutrient agent.
37. A bioremediation composition of claim 35 which if formulated for controlled release.
38. A method of bioremediation, comprising the step of exposing a sample to a

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composition of any one of claims 35-37.

39. A method of claim 38, wherein the sample is contaminated with radionuclides.

40. A method of claim 39, wherein the composition is released into a waste site.

41. A radiation resistant bacterium of any one of claims 1-4 and 16-24, wherein the bacterium is selected from the group consisting of *D. radiodurans*, *D. radiopugnans*, *D. grandis*, *D. proteolyticus*, *D. murrayi*, *D. geothermalis*, and *D. radiophilus*.

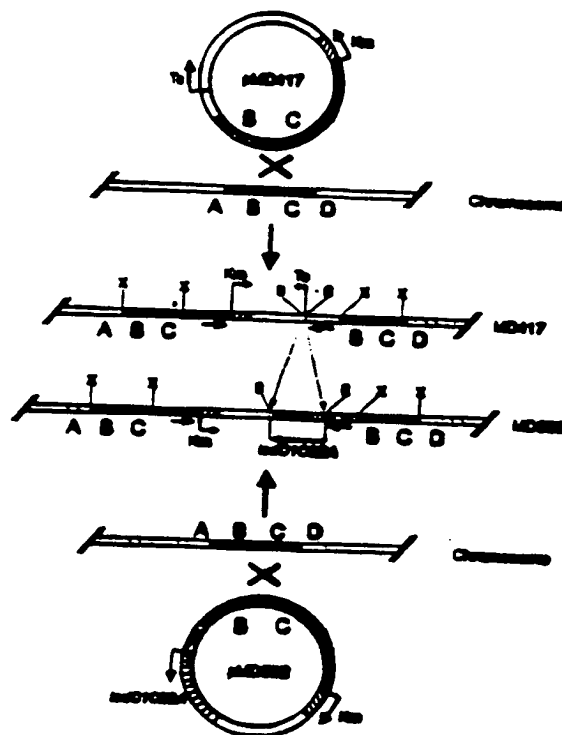


Figure 1

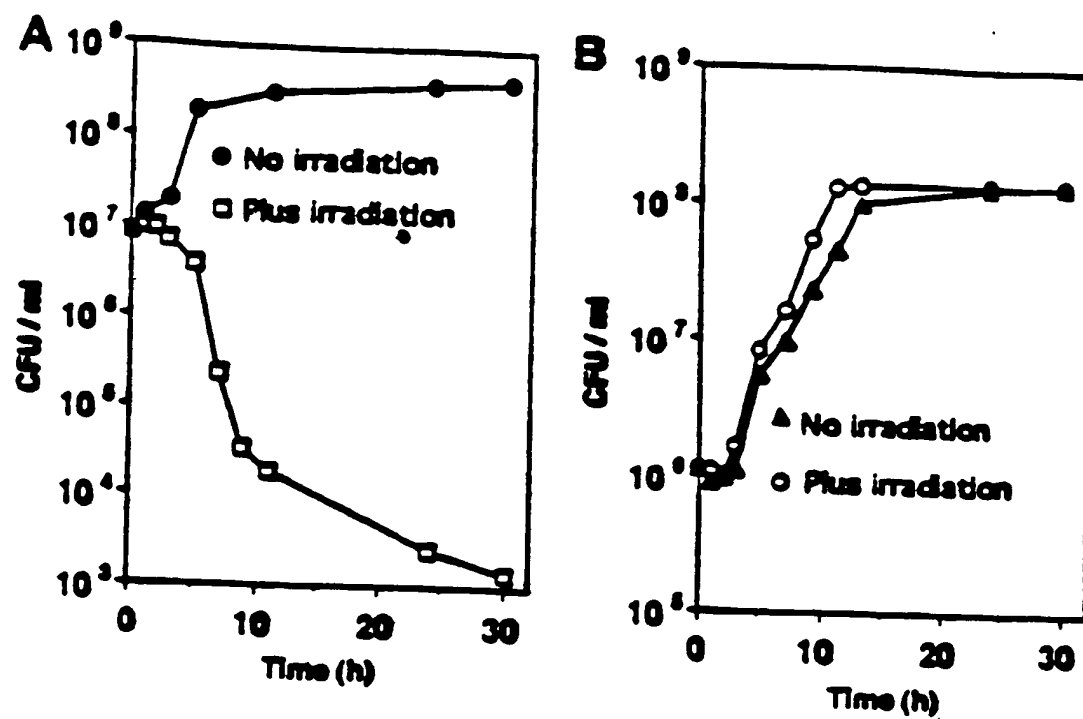


Figure 2

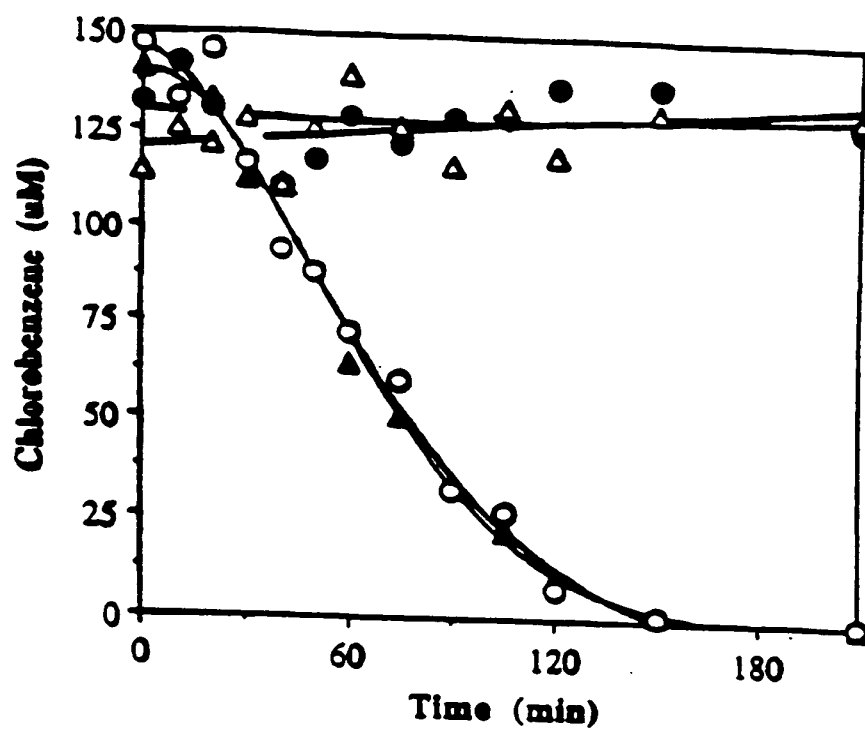


Figure 3

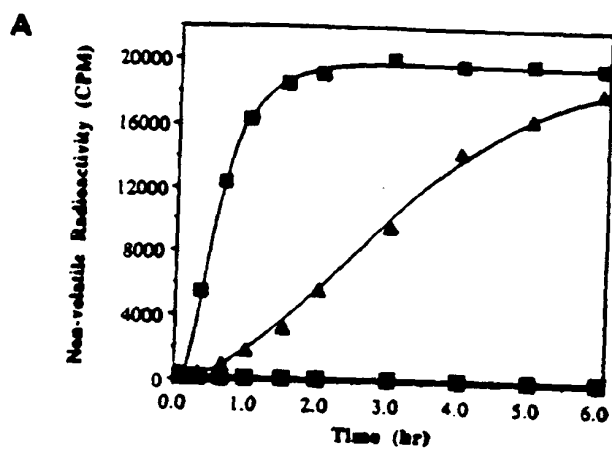


Figure 4

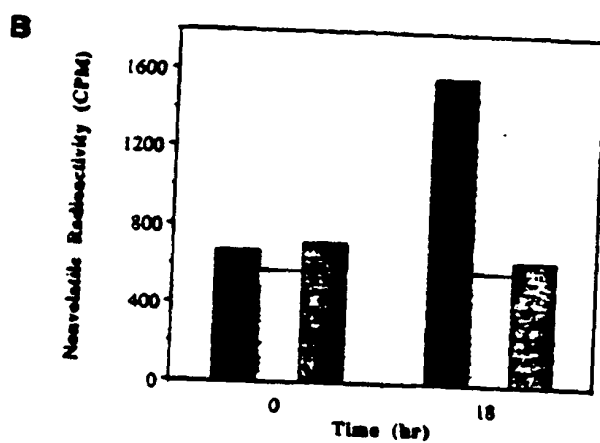


Figure 5

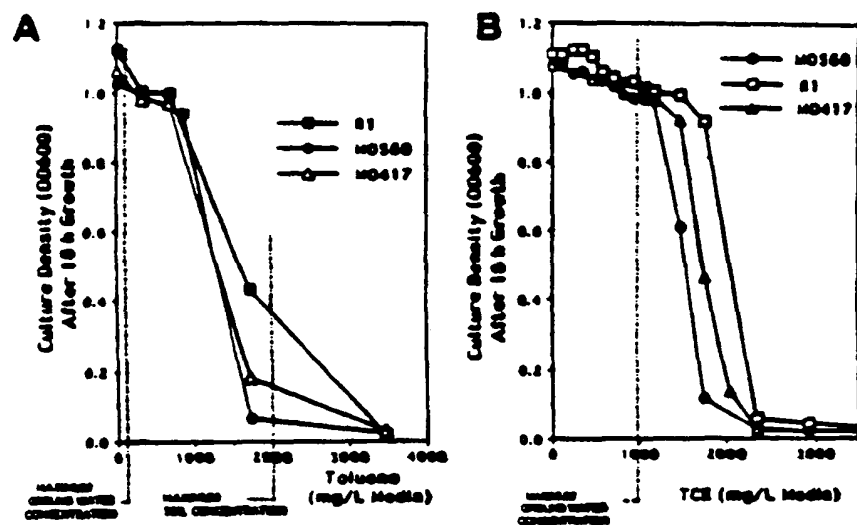
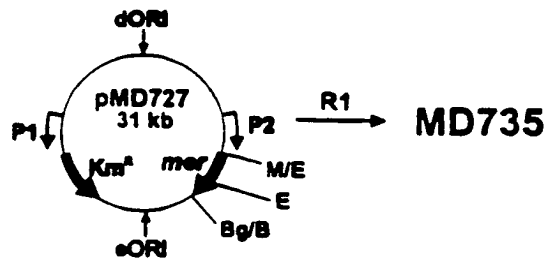


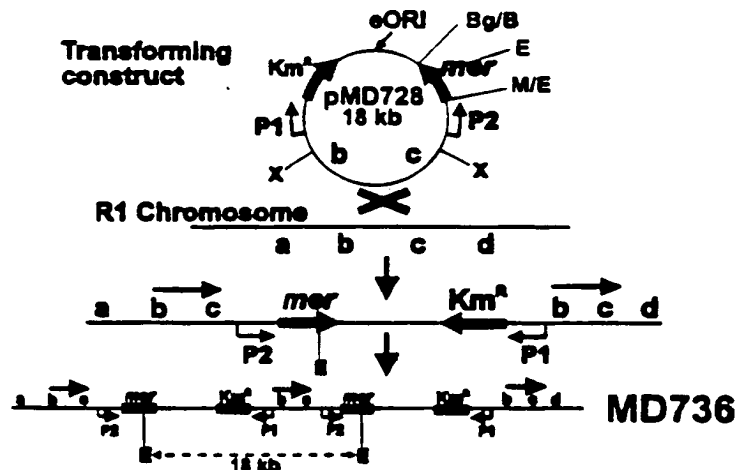
Figure 6



A



B



C

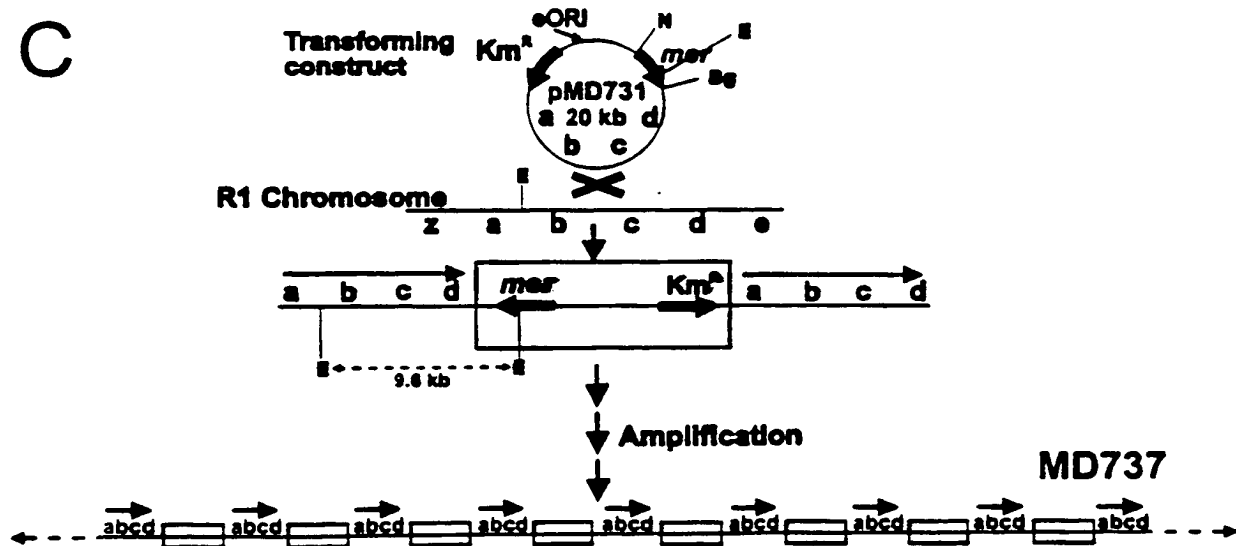


Figure 7

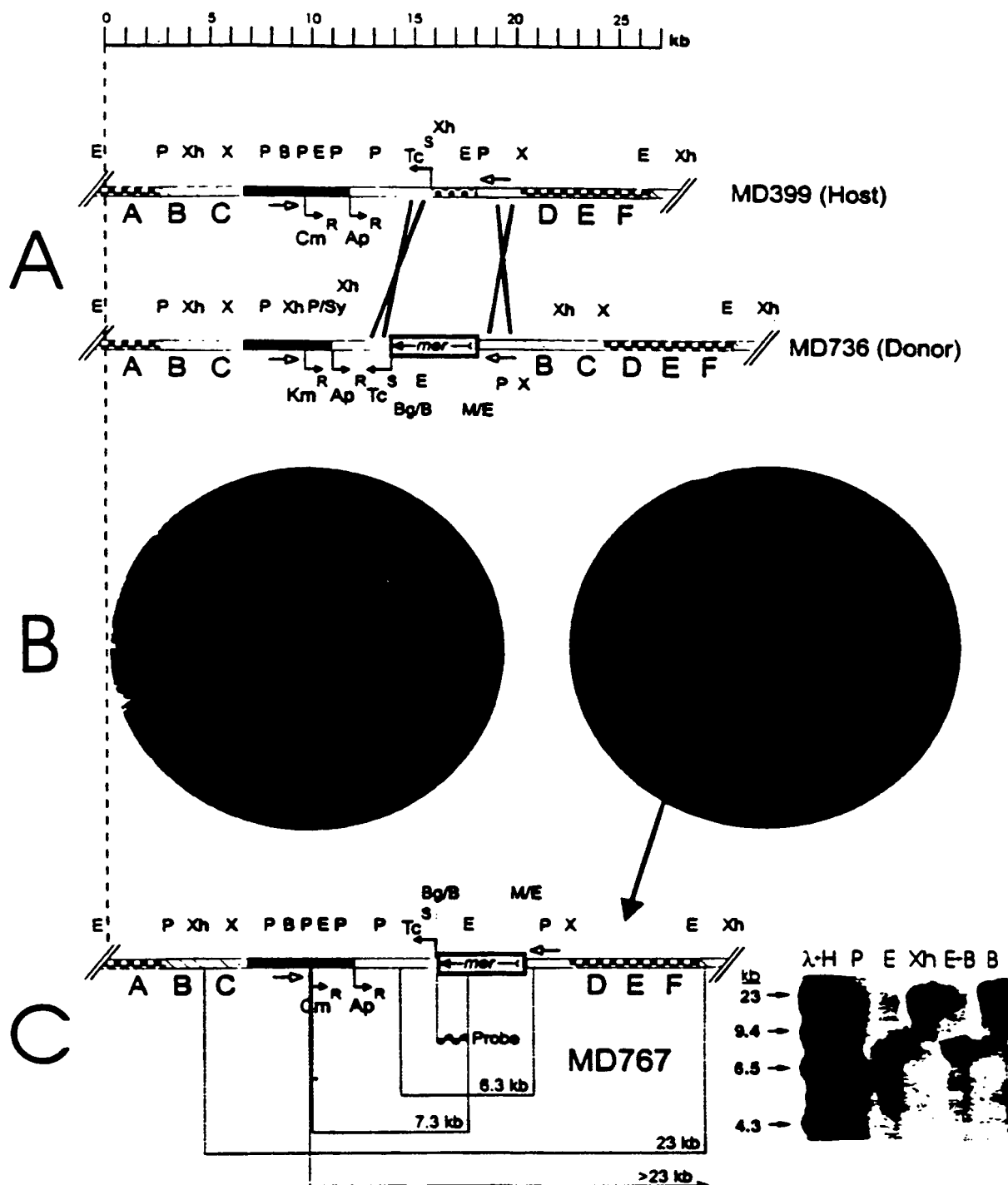


Figure 8

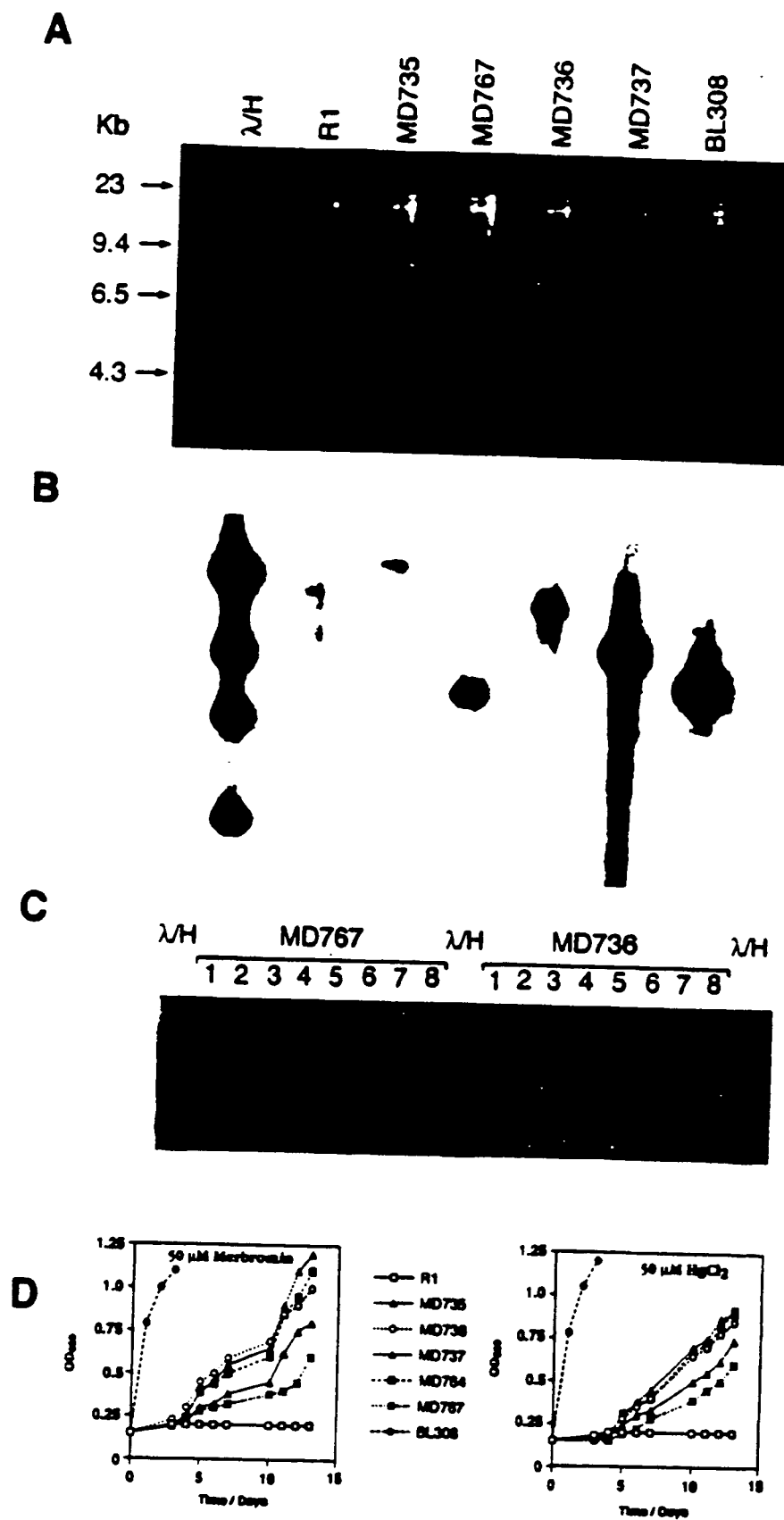


Figure 9

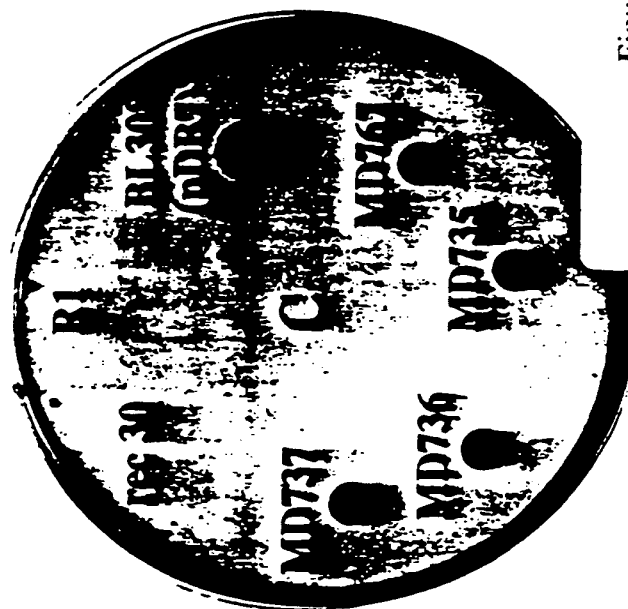
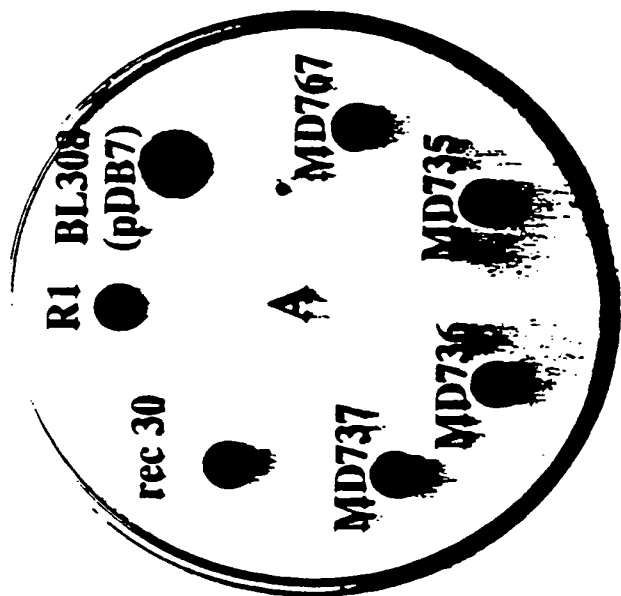
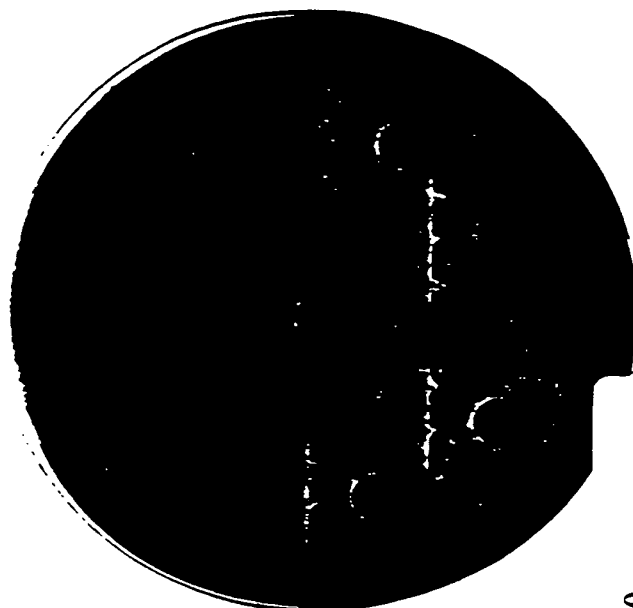
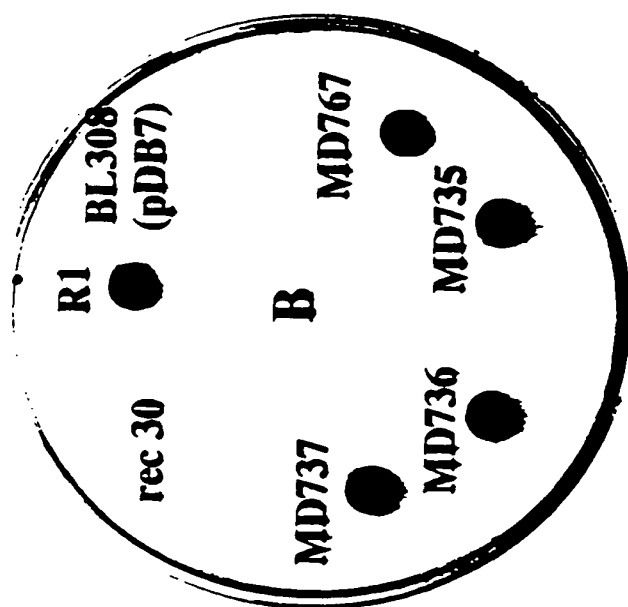


Figure 10

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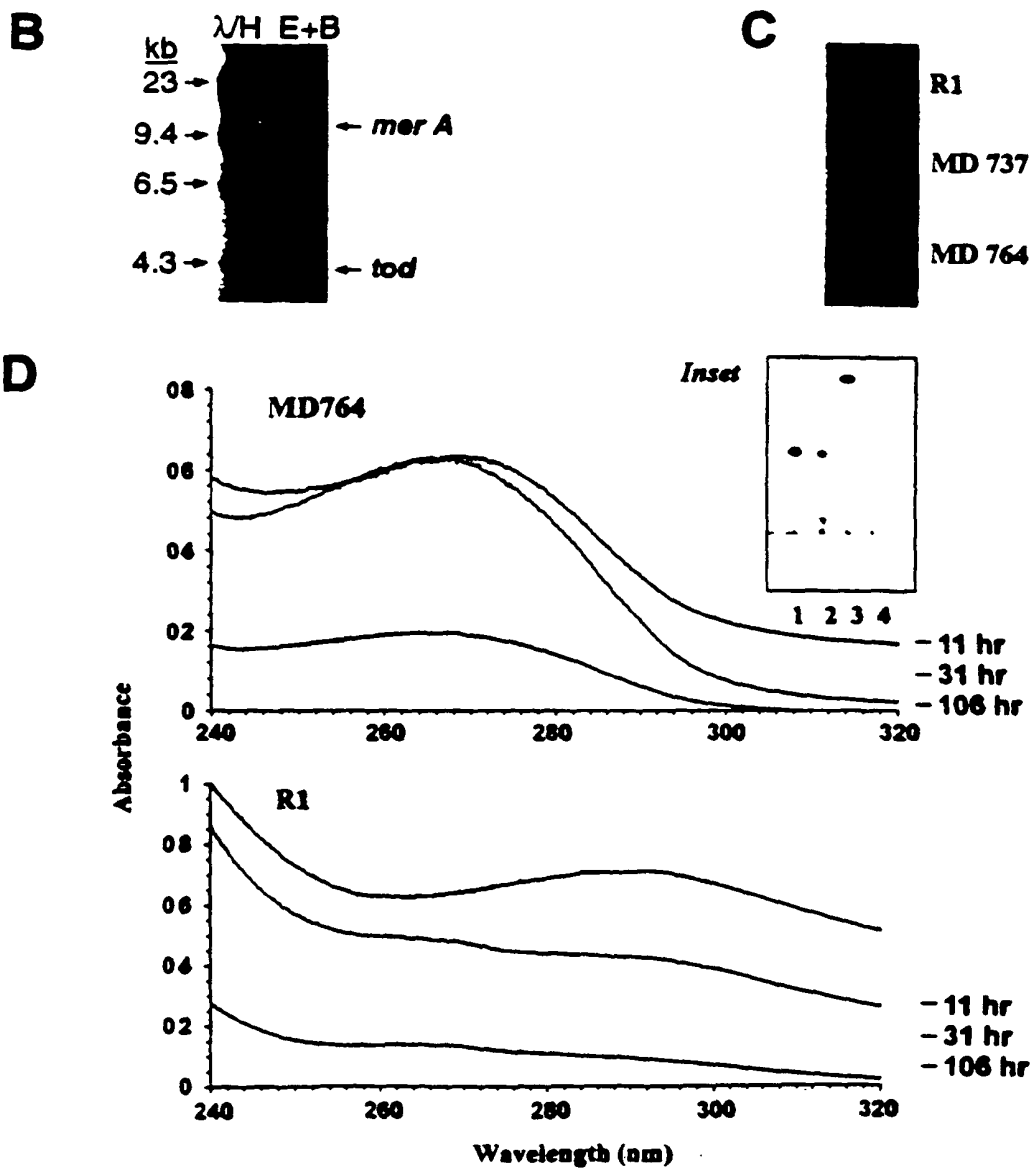
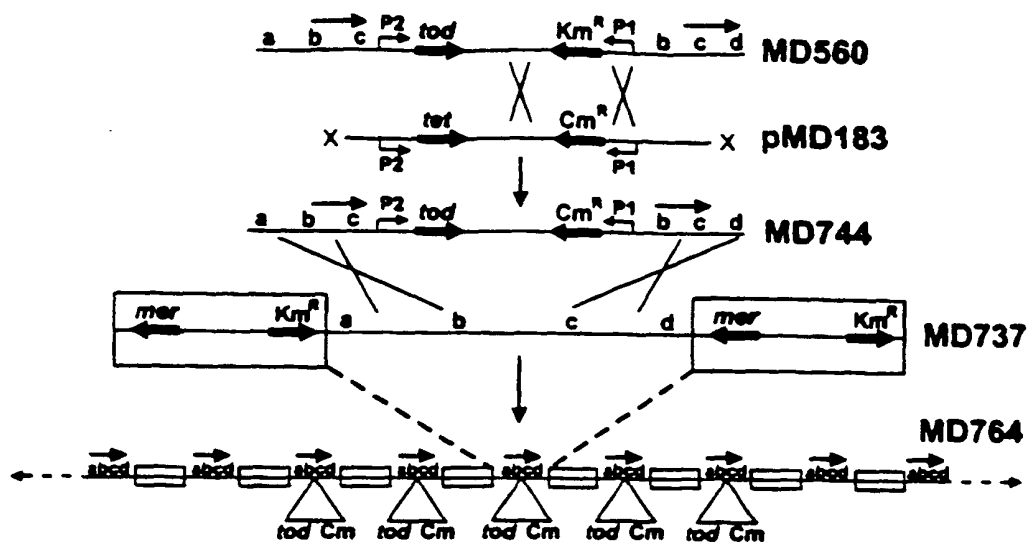


Figure 11

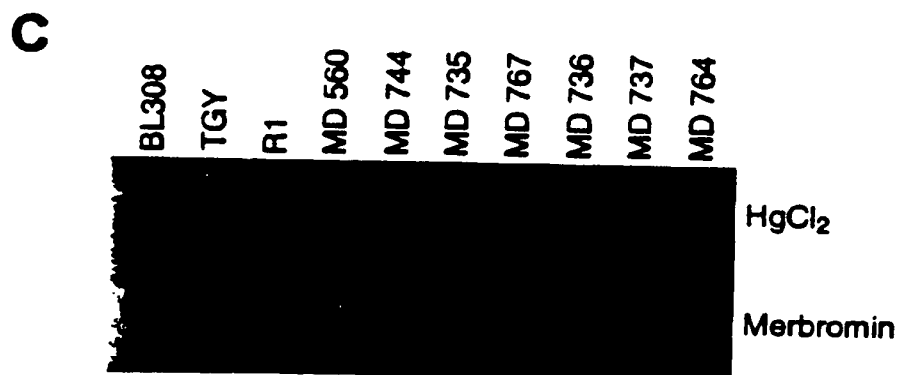
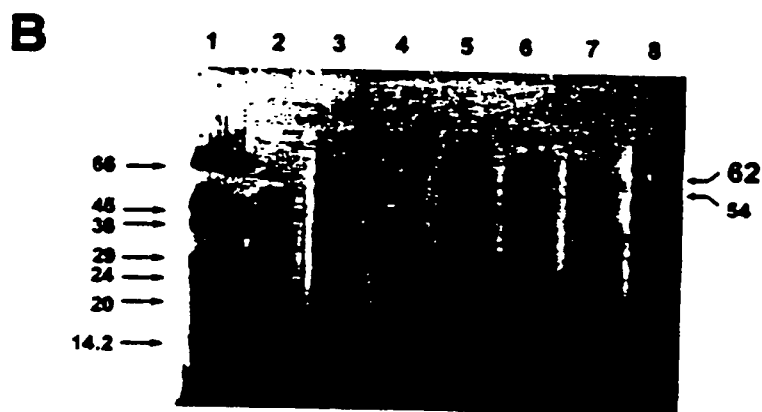
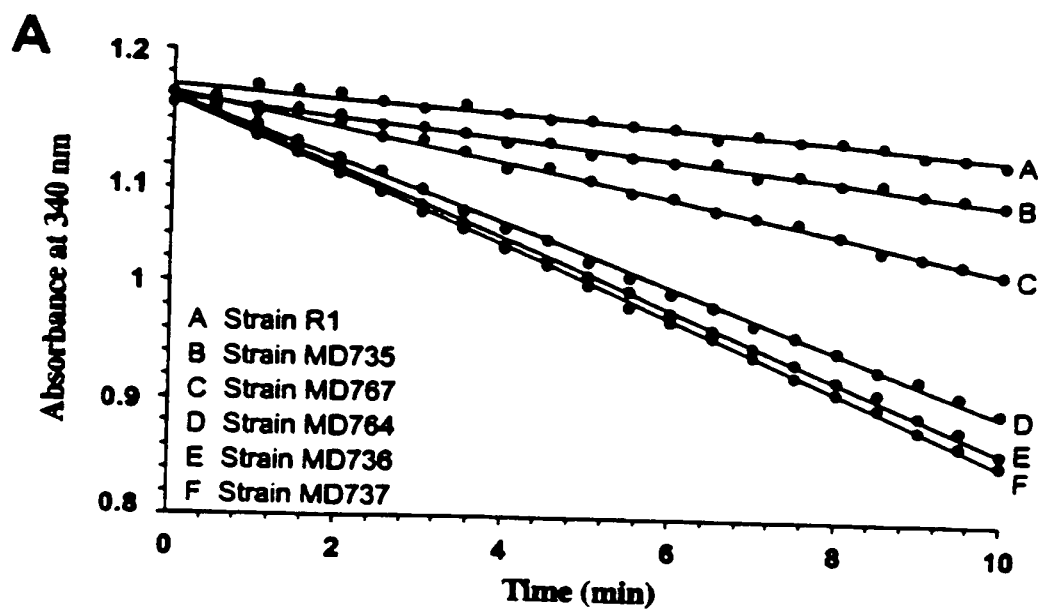


Figure 12

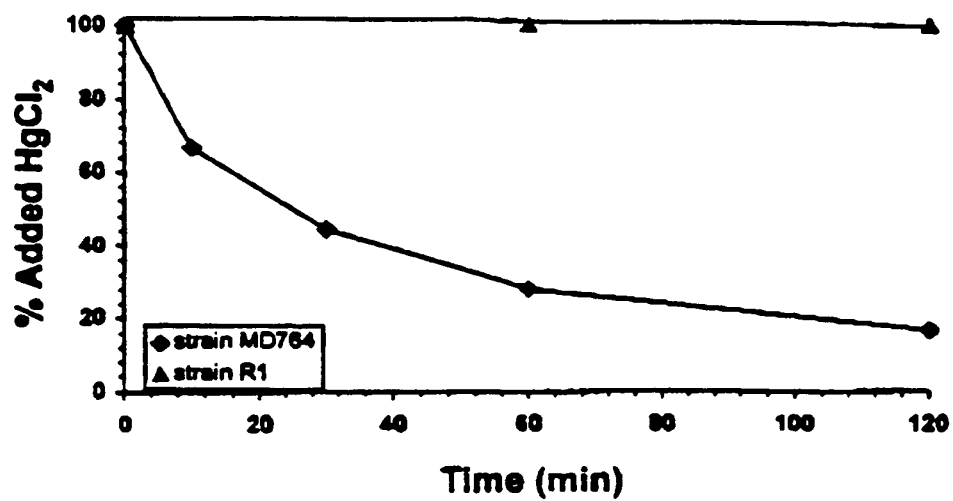


Figure 13

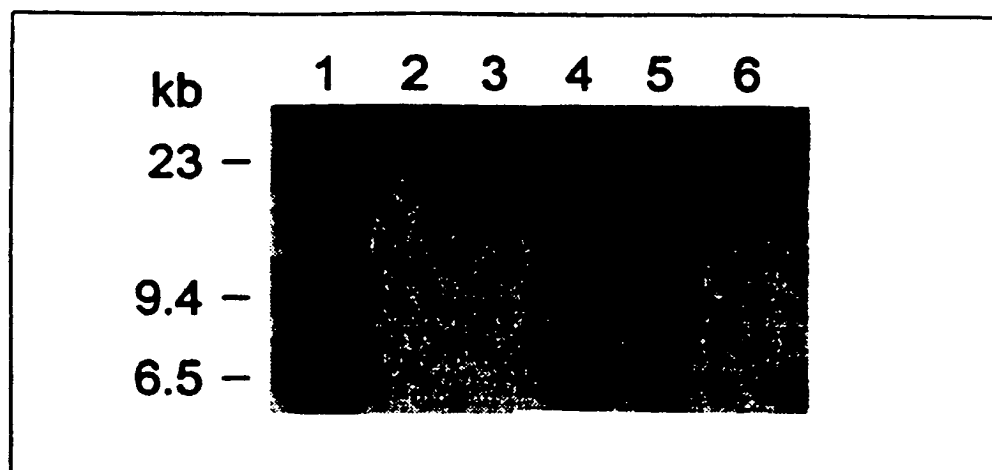


Figure 14